Microbial community analysis of constructed wetlands treating effluent from a land-based marine fish farm.

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Abstract

The use of constructed wetlands to treat wastewater from fish farms has the potential to reduce the environmental impact of the aquaculture industry. In order to gain a greater understanding of the processes occurring within a newly constructed wetland at a commercial marine fish farm, nitrogen removal and bacterial communities were studied in model wetlands and the fish farm wetland.

The limits of nitrification capacity in a vertical trickle flow model system were tested by dosing with aquaculture wastewater supplemented with increasing amounts of ammonium chloride. Greater than 97% ammonia removal was observed with ammonia concentrations of up to 358ppm. At higher concentrations of ammonia a lower percentage of ammonia removal occurred, and nitrite accumulation was observed. A decrease in the number of detected bacterial OTUs (as measured by 16s rRNA T-RFLP) was detected concurrent with the reduction in the percent of ammonia removed. T-RFLP analysis of the ammonia mono-oxygenase gene showed a clear successional pattern of three different ammonia oxidizing bacterial OTUs (belonging to the *Nitrosomonas* Nm143 lineage *N.oligotropha/N.ureae* lineage and *N.aestuarii/N.marina* lineage).

Lab-scale wetlands were used to investigate the effect of flood/drain cycles on nitrogen removal. When the multiple cycles were used, the concentrations of ammonia and organic nitrogen were lower after treatment in the flood/drain wetlands than in permanently submerged wetlands. However, the concentrations of nitrites and nitrates were higher in the flood/drain wetlands. Elevation of nitrate concentration could be prevented by shortening the drainage period. Subsequent work on the fish farm wetland also showed that flood/drain cycles improved ammonia removal, but reduced nitrate and nitrite removal. The total bacterial communities in the submerged wetlands showed a greater degree of similarity to each other than those in the flood/drain wetlands. The ammonia oxidizing bacterial communities in the flood/drain wetlands were dominated by bacteria belonging to the *Nitrosomonas aestuarii/N.marina* lineage, and the submerged wetlands were dominated by a bacterial OTU that was unidentified by T-RFLP.

Nitrosomonas aestuarii/N.marina was the dominant ammonia oxidizing bacteria during the first 17 months of operation of the fish farm wetland. The abundance of other ammonia oxidizing OTU showed seasonal variation. The total bacterial community did not show clear temporal or spatial patterns of variation. Effective nitrogen removal was seen in the wetlands with the exception of one pair of cells which began to experience elevated ammonia concentrations after about 15 months. It was shown that introducing flood/drain cycles to this wetland could rapidly improve performance and prevent ammonia accumulation.

Abbreviations

AOB	Ammonia oxidizing bacteria
bp	base pairs
DDGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleoside triphosphate
EDTA	Ethylene diamine tetraacetic acid
HFW	Horizontal flow wetland
HLR	Hydraulic loading rate
HRT	Hydraulic residence time
IPTG	Isopropyl-thiogalactoside
L	Litre
M	Molar
min	minute(s)
µ	micro
NOB	Nitrite oxidizing bacteria
OTU	Operational taxonomic unit
Org-N	Organic nitrogen
PCR	Polymerase chain reaction
ppt	parts per thousand
ppm	parts per million
rDNA	ribosomal DNA
rpm	Revolutions per minute
rRNA	ribosomal Ribonucleic acid
SE	Standard error

SLS	Sample loading solution
TAN	Total ammoniacal nitrogen
TBE	Tris Borate EDTA
TIN	Total inorganic nitrogen
TKN	Total Kjeldahl nitrogen
TRF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
VFW	Vertical flor wetland
X-gal	*5*-bromo-chloro-*3*-indolyl-β-D-galactoside

Contents

Chapter One

Introduction

1.1 Microbial ecology of wetland bacterial communities

1.1.1 Molecular methods of describing bacterial communities

Constructed wetlands are commonly used to the treat wastewater from a variety of sources. Despite their widespread use, the full complexity of the biological mechanisms of pollutant removal, such as microbial activity, plant-microbe interactions and the role of abiotic factors are not completely understood and remains the focus of much research. A clear picture of the microbial community structure and functioning is important so that the performance of wetlands can be improved (Wagner & Loy, 2002). It has been reported (e.g. by Amann *et al.*, 1995) that typically less than 1% of the bacterial cells from environmental samples can be cultured (which has often led to a focus on using culture-independent methods of assessing microbial diversity). However, since this figure is a measure of the proportion of cells that can be cultured, not a measure of the diversity of organisms that can be cultured, the often held view that less than 1% of bacterial species can be cultured is wrong (Rothschild, 2006). Cultivation based and cultivation-independent methods will identify different components of the community and both have their place in describing microbial diversity (Donachie *et al.*, 2007).

Molecular methods such as terminal restriction fragment analysis (T-RFLP), automated ribosomal intergenic spacer analysis (ARISA) and diffusion gradient gel electrophoresis (DGGE) can be used to profile microbial communities (Fisher & Triplett, 1999; Hartmann *et al.*, 2005). All these

techniques can be used even when there is limited prior knowledge of the organisms likely to be present. They rely upon the use of PCR primers that target conserved sequences for a particular phylogenetic group. The community can then be analysed by separating different taxa by utilizing differential electrophoretic migration rates. In T-RFLP a section of a gene (most commonly the 16S rRNA gene) is amplified using a fluorescent labelled primer and then digested with a restriction endonuclease. With this method length variation in the terminal restriction fragment (with the fluorescent primer attached for detection) is the basis for identifying different taxa (there is no way to identify whether individual bands represent a single species or a group of species, so the term operational taxonomic unit (or OTU) is used to refer to a group that is represented by a single electrophoresis band). Like T-RFLP, denaturing gradient gel electrophoresis (DGGE) and automated ribosomal intergenic spacer analysis also rely on universal primers, but use reduced mobility of denatured DNA and length heterogeneity respectively to separate different OTUs. Although PCR techniques such as these have many advantages over cultivation based methods, such as speed of analysis and ability to identify unculturable bacteria, there are some minor disadvantages such as the potential for PCR bias (Osborn *et al.*, 2000), difficulties with interpreting heterogeneity in ribosomal RNA (*rrn*) operon copy number and the inability to identify and exclude long surviving allocthanous DNA (Purkhold *et al.*, 2000), which need to be taken into consideration.

1.1.2 Bacterial communities in wastewater and polluted fish farm sediments

Much of the focus in microbiological studies of wastewater treatment is on the organisms that carry out specific chemical conversions, such as oxidation of ammonia or reduction of nitrite. However communities obviously consist of more than the few species of bacteria that are carrying out useful industrial processes. There are many, still mostly unknown bacteria that carry out important supporting functions in wastewater treatment, for example biofilm formation, affecting flocculation, bulking sludge and carbon cycling (Kadlec & Knight, 1996). Additionally, there are complex relationship between some heterotrophic bacteria and autotrophic nitrifying bacteria; high levels of organic carbon increases heterotrophic ammonia assimilation at the expense of autotrophic nitrification, particularly ammonia oxidation (Hanaki *et al.*, 1990; Verhagen & Laanbroek, 1991). However in carbon limited media a wide range of heterotrophic bacteria form close spatial relationships with clusters of nitrifying bacteria in order to utilise soluble microbial products they produce, this kind of association appears to benefit both the heterotroph and the nitrifier (Clark & Schmidt, 1965; Jones & Hood, 1980; Kindaichi *et al.*, 2004).

Estimating the bacterial diversity in an environment is a difficult task, and will vary according to the method used. Analysis of bacterial communities in wastewater plants estimated the minimum number of species in wastewater treatment plants to be between 17-268 (Wagner & Loy, 2002). Two studies on fish farm sediments have indicated that organic pollution reduces diversity. One study based on a DNA reassociation technique estimated that marine fish farm

sediments were much less diverse than nearby pristine sediment (only 6-13 different genomes for fish farm sediment compared to 11400 for pristine sediment). The number of genomes increased to 1700 in sediment from a farm abandoned four years earlier (Torsvik *et al.*, 1998). In a second study using clone libraries, bacterial diversity increased in sediment beneath fish farm cages that had been left empty for three months. Furthermore the communities beneath the cages were distinct from unpolluted reference sediment, not simply a reduced subset of them (Bissett *et al.*, 2006). The authors believed higher species richness in their study (compared to the Torsvik *et al.* study) could be due to lower organic loading. The clone library approach allowed changes in fish farm sediment community structure to be observed; in common with other studies on marine sediment *δ-* and γ-*proteobacteria* and the *Cytophaga-Flavobacteria-Bacteroidetes* group were dominant in all samples, but in addition α- and *ε-proteobacteria* were also dominant in fish farm sediments. A review of wastewater treatment and activated sludge systems revealed α-, β- and γ-proteobacteria, bacteriodes and actinobacteria to be the most commonly occurring bacterial groups (Wagner *et al.*, 2002).

1.2 The nitrogen cycle

Removal of high concentrations of ammonia, nitrates and nitrites as well as other pollutants from wastewater is an environmentally desirable aim of water

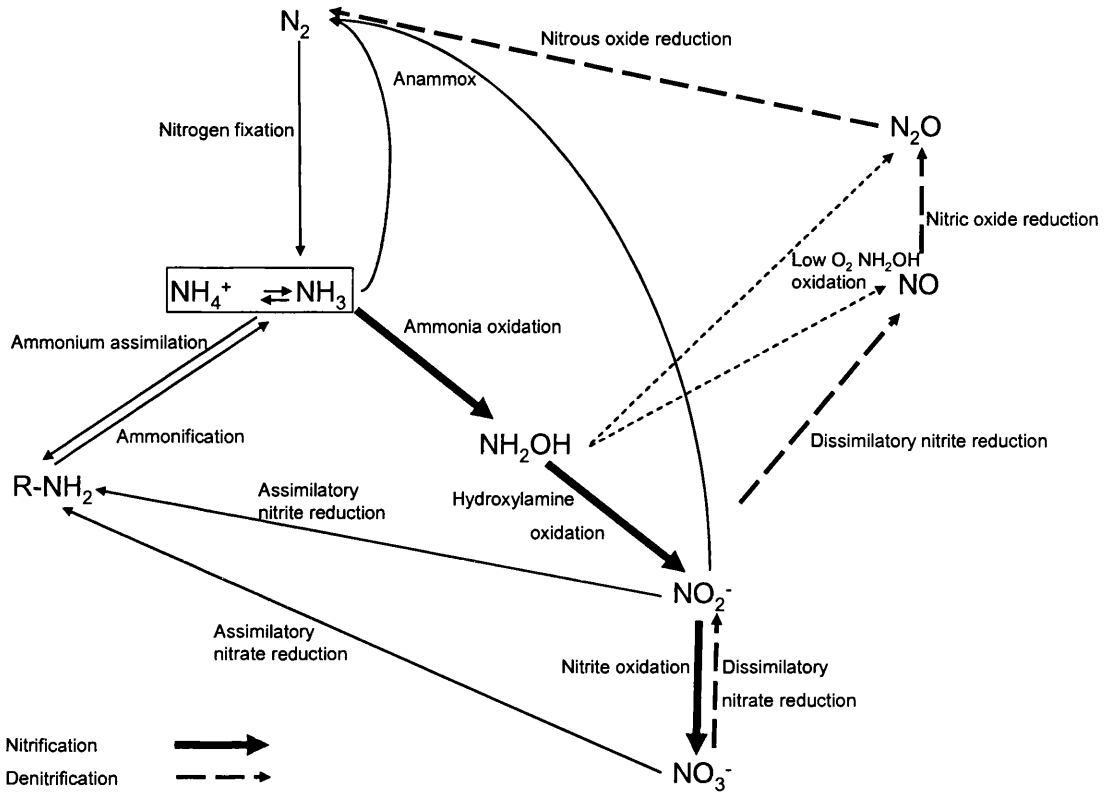


Figure 1.1. Some of the bacterially mediated processes in the nitrogen cycle (adapted from (Richardson, 2000; Robertson & Groffman, 2006; Ye & Thomas, 2001). The nitrification and denitrification pathways are marked in bold solid and bold dashed arrows respectively.

treatment. Accumulation of ammonia and nitrite can be toxic to aquatic life and nitrates can contribute to eutrophication of water bodies as well as pose a health risk if they contaminate water supplies. Incomplete denitrification produces nitric oxide (NO) and nitrous oxide (N_2O), which are greenhouse gases. Some of the complexity of the nitrogen cycle is summarized in Fig. 1.1. The removal of nitrogenous pollutants in waste is usually considered as two processes; nitrification and denitrification. Nitrification is the conversion of ammonia to

nitrate, and denitrification the conversion of nitrate to nitrogen gas. The first process is traditionally considered to be carried out predominantly by aerobic chemolitho-autotrophic bacteria capable of using inorganic nitrogen as an energy source and the latter by a wide variety of heterotrophic anaerobic microorganisms including archeobacteria, proteobacteria, gram positive eubacteria and fungi (Ye & Thomas, 2001). However a much more complex situation is beginning to emerge with the discovery of nitrifying archaea, anaerobic ammonia oxidizers (anammox bacteria) and greater understanding of the flexibility of the autotrophic ammonia oxidizers, all of which will be discussed in the following sections.

1.3 Nitrification by autotrophic bacteria

Nitrification proceeds in two steps, ammonia oxidation (the conversion of ammonia to nitrite via hydroxylamine) and nitrite oxidation (the conversion of nitrite to nitrate) each of which is carried out by a separate group of organisms.

1.3.1 Organisms responsible for ammonia oxidation

Most species of autotrophic ammonia oxidizing bacteria belong to the β-proteobacteria class and are placed in the family Nitrosomonadaceae within the order Nitrosomonadales. Bergey's manual currently recognises 16 species of autotrophic nitrifying bacteria (Garrity *et al.*, 2004). Divided into two genera

Nitrosomonas and *Nitrosospira*. The genus *Nitrosomonas* is divided into six lineages and the genus *Nitrosospira* into five subgroups or clusters (Figure 1.2). The evidence for these divisions is weaker in *Nitrosospira,* but appears to be ecologically relevant (Prosser, 2007). The three named *Nitrosospira* species all belong to the same subgroup. Among the nitrosomonads, the Nm143-lineage has been identified from environmental clones and has no cultured representative (Purkhold *et al.*, 2003). Ammonia oxidizing γ-proteobacteria, belong to the genus *Nitrosococcus* (family Chromatiales, family Chromatiaceae), and are believed to be to limited to marine environments (Koops & Pommerening-Roser, 2001).

Studies on wastewater treatment systems (including treatment wetlands) and aquaria usually reveal an ammonia oxidizing community dominated by *Nitrosomonas* species (e.g. Burrell *et al.*, 2001; Egli *et al.*, 2003; Hovanec & DeLong, 1996; Layton *et al.*, 2005; Limpiyakorn *et al.*, 2005; Otawa *et al.*, 2006; Rowan *et al.*, 2003; Silyn-Roberts & Lewis, 2003; Wagner & Loy, 2002) although some studies (more often in wetlands) do show *Nitrosospira* to be dominant (Austin *et al.*, 2003; Gorra *et al.*, 2007 in wetlands; Schramm *et al.*, 1998 in a nitrifying reactor). One study found *Nitrosomonas* to be dominant all parts of a dairy wastewater treatment system except the wetland where *Nitrosospira* dominated (Ibekwe *et al.*, 2003). In a study on the trickling filter biofilm within a recirculating marine aquaculture system the Nm143-lineage was found to be most abundant, followed by *Nitrosomonas marina*-like bacteria (Foesel *et al.*, 2008). In an aquaculture study, proximity to the fish cages affected the

community composition. The ammonia oxidizer community structure was shown to vary with distance from a marine fish farm; all sites had the same *Nitrosomonas* and *Nitrosospira* species except the most polluted sites in which an additional *Nitrosomonas* species appeared (McCaig *et al.*, 1999), indicating that the microbial community structure reflects the local environment.

1.3.2 Biochemistry of ammonia oxidation

The substrate for this oxidation reaction is believed to be molecular ammonia rather than ammonium ions (Wood, 1986). Some ammonia oxidizers have urease activity which allows them to derive ammonia from urea that can subsequently be oxidized (Kowalchuk & Stephen, 2001). The oxidation involves two enzymatic steps. The conversion of ammonia to hydroxylamine, catalysed by ammonia mono-oxidase (AMO) and the conversion of hydroxylamine to nitrite, catalysed by hydroxylamine oxido-reductase (HAO).

$$2H^+ + NH_3 + 2e^- + O_2 \rightarrow NH_2OH + H_2O \qquad (1.1)$$

$$NH_2OH + H_2O \rightarrow HNO_2 + 4e^- + 4H^+ \qquad (1.2)$$

Molecular oxygen acts as an electron acceptor in the oxidation of ammonia to hydroxylamine (Equation 1.1). No accumulation of hydroxylamine has been observed in bacteria, and so it is assumed to be immediately oxidized

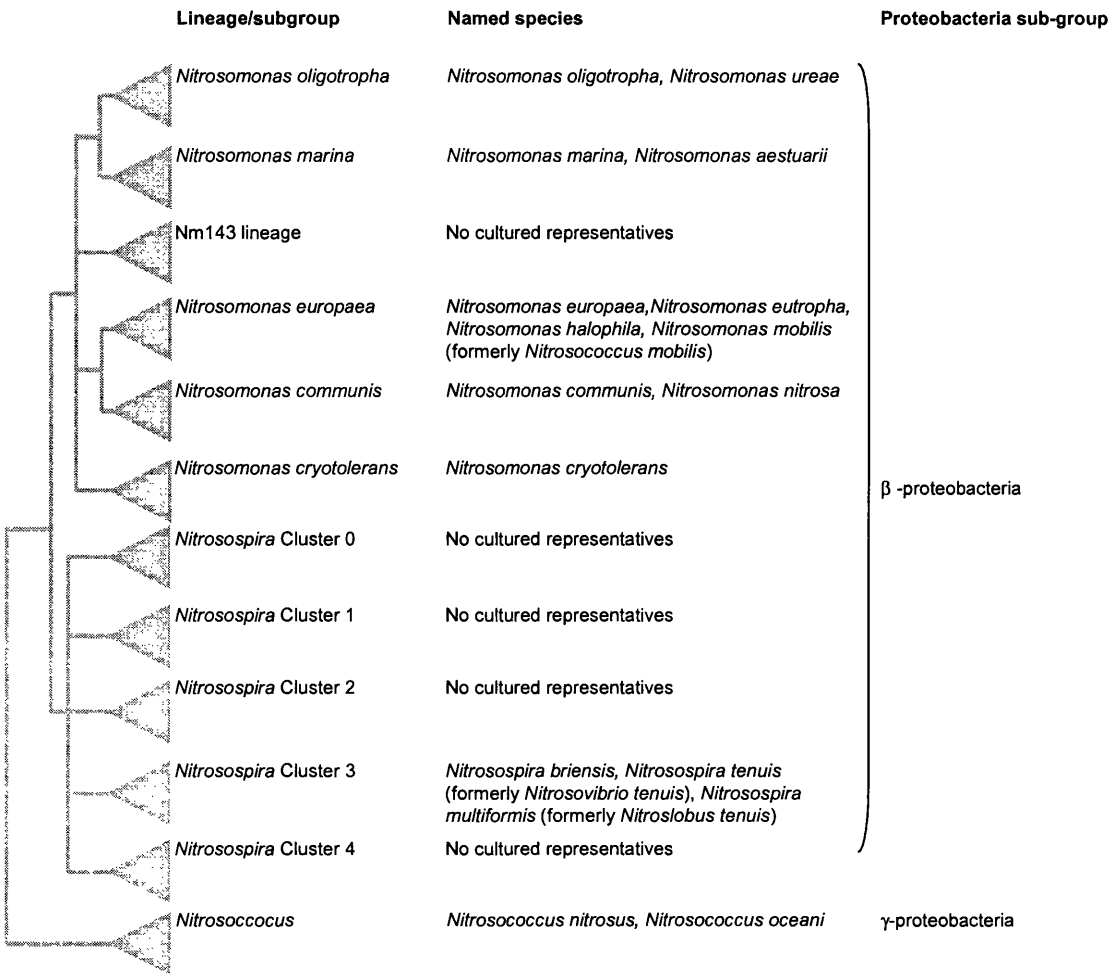


Figure 1.2. Schematic dendrogram showing relationships between all recognised proteobacterial autotrophic ammonia oxidizing bacteria and major lineages (Koops & Pommerening-Roser, 2001; Purkhold *et al.*, 2000; Purkhold *et al.*, 2003)

to nitrite (Wood, 1986). Hydroxylamine is the primary energy generating molecule in the oxidation of ammonia and the function of the AMO enzyme is to produce the hydroxylamine from which electrons (and protons) are produced. The oxidation of hydroxylamine (Equation 1.2) proceeds by electrons transfer from hydroxylamine to the oxygen molecule in water (Hooper *et al.*, 2004).

Autotrophic ammonia oxidizers utilize a pathway in which only the second step of ammonia oxidation (Equation 1.2) is energy producing. Two of the four electrons produced are used up in the production of hydroxylamine. Most of the remaining electrons pass to terminal electron acceptors and are used in reduction of toxic substances, nitrite or sulphate, or are used in the oxygenation of organic molecules. Subsequently only 0.35 out of the four electrons produced in each reaction go towards the generation of NADH and energy production (Hooper *et al.*, 2004). Most of the energy generated by autotrophic ammonia oxidation goes into fixation of carbon dioxide, which explains the slow growing nature of nitrifiers.

1.3.3 Ecology of AOB

Chemolithoautotrophic ammonia oxidizing bacteria are found in virtually all environments persisting in a wide range of temperatures, pHs and oxygen concentrations (Schmidt *et al.*, 2002). Three of the *Nitrosomonas* species are obligate halophiles (*N.marina, N.aestuarii and N.cryotolerans*). *Nitrosomonas europaea* is halotolerant while other nitrosomonad and *Nitrosospira* species have no salt requirement (Koops & Pommerening-Roser, 2001)

Since ammonia oxidizing bacteria rely on ammonia as their sole energy source they have evolved a range of mechanisms and that allow them to cope with natural fluxes of ammonia and oxygen in the environment. Understanding these is helpful in understanding the behaviour of bacteria in water treatment systems. AOB are able to survive for long periods (at least one year) in the

absence of ammonia, and recover rapidly once ammonia is added. Long starvation periods are survived by switching to a low energy metabolism with low decay rates and low maintenance requirements (Geets *et al.*, 2006). The speed of recovery depends on the duration of starvation and the species (with *Nitrosomonas europaea* recovering faster than *N. oligotropha or Nitrosospira briensis*) (Bollmann *et al.*, 2002). The slow responding species had the ability to continue oxidizing ammonia at lower concentrations than the fast responding species. This suggests that there is niche differentiation, with fast responding bacteria adapted to environments with unreliable ammonia input, but slow responding species adapted to environments with constantly low ammonia. Under low oxygen conditions ammonia oxidizing bacteria may switch to denitrification or anaerobic ammonia oxidation. They are able to reduce nitrite (using hydroxylamine or organic matter as electron donors) to dinitrogen or nitrogen oxides whilst simultaneously oxidizing ammonia (Bock *et al.*, 1995). This process occurs in aerobic conditions, but appears to be increased in anaerobic conditions (Ritchie & Nicholas, 1972). In low oxygen conditions shorter partial denitrification pathways appear to be favoured (Kester *et al.*, 1997; Poth, 1986). The capability to denitrify is thought to be a protective mechanism to avoid nitrite poisoning. An additional effect of gas production in denitrification is to provide a transport mechanism that allows movement to favourable environments. Philips (2002) observed that nitrifiers moved from sediment to overlying surface waters and hypothesised that in the nitrifier, denitrification occurs in the low oxygen conditions of the sediment. The gaseous

products of denitrification form bubbles which adhere to the bacterial cell surface. The increased buoyancy allows the bacteria to float from their low oxygen environment to the more aerobic surface where they can resume nitrification. Additionally in anaerobic conditions ammonia oxidation is possible using nitrogen tetroxide (N_2O_4) in place of oxygen as the electron acceptor, also producing nitric oxide (Geets *et al.*, 2006).

1.4 Other organisms involved in nitrification

1.4.1 Ammonia oxidation by archaea

Evidence that oxidation of ammonia by archaea was possible came from the identification of an *amoA*-like gene on an archaeal-associated scaffold, in a metagenome study of the Sargasso sea (Venter *et al.*, 2004), and later from calcarious grassland soil (Treusch *et al.*, 2005). The first aerobic ammonia oxidizing archaea was cultivated in 2005, it was identified as a member of the *Crenarchaeota* phylum and designated as *Candidatus* Nitrosopumilus maritimus (Konneke *et al.*, 2005). Ammonia oxidation by archaea is now thought to be widespread in a range of natural and industrial environments, and occurs in a much more phylogenetically diverse group of organisms than is seen in the bacteria. In certain environments archaea may be more important for nitrification than bacteria (Francis *et al.*, 2005; Wuchter *et al.*, 2006). *Candidatus* Nitrosopumilus maritimus has recently been identified as occurring

at low abundances (<0.1% of all cells in biofilm) in a recirculating marine aquaculture trickle filter (Foesel *et al.*, 2008).

1.4.2 Anaerobic ammonia oxidizing (anammox) bacteria

Unlike nitrification in archaea which was deduced following the identification of functional genes, the existence of microorganisms with the ability to oxidize ammonia under anaerobic conditions was suspected before the discovery of the organisms or genes responsible. Observational data from the 1960s suggested the possibility of anaerobic microbial oxidation (Francis *et al.*, 2007), and the occurrence of "chemosynthetic bacteria that oxidize ammonia to nitrogen with O_2 or nitrate as oxidant" was formally hypothesised in 1977 (Broda, 1977). The first organisms (named *Candidatus* Brocadia anammoxidans) were isolated in 1992 from a wastewater plant denitrifying reactor in Delft, Netherlands (Mulder *et al.*, 1995). Nitrite (rather than oxygen or nitrate) is the preferred electron acceptor for these organisms, and hydroxylamine and hydrazine are produced as intermediate products (Jetten *et al.*, 2002). Ammonia and nitrite combine to produce dinitrogen gas and water as follows.

$$NH_3 + HNO_2 \rightarrow N_2 + 2H_2O \qquad (1.3)$$

There are currently four known genera of anammox bacteria *Candidatus* 'Brocadia', 'Kuenen', 'Scalindula' and 'Anammoxoglobus' all belonging to the Planctomycetales (Francis *et al.*, 2005). Initially these organisms were

considered to be restricted to industrial environments but recent research has indicated that they are widespread in the marine environment (Schmid *et al.*, 2007). Estimates of the contribution of anammox to nitrogen losses from marine environments range from 20 to 67% (Francis *et al.*, 2007). Anammox can be detected in freshwater constructed wetlands that have been designed to promote that process (e.g. Dong & Sun, 2007), but the contribution of anammox to ammonia removal in constructed wetlands in general is not clear.

1.4.3 Heterotrophic oxidation of ammonium compounds

Although autotrophic nitrification has been regarded as the predominant method of ammonia oxidation, it has been recognised for some time that many heterotrophic organisms can produce nitrite from ammonium or organic compounds, although this does not appear to be associated with energy production (Hagopian & Riley, 1998). A significant proportion of actinomycetes (27% of tested strains), bacteria (26%) and fungi (17%) can produce nitrite from organic nitrogen sources in laboratory cultures (Eylar & Schmidt, 1959). Another study found that a similar percentage of actinomycetes (30%) produced nitrite from inorganic ammonium (Hirsch *et al.*, 1961). Nitrifying heterotrophs have also been identified in enviromental samples, including wastewater treatment systems, and may be important in wetlands that are operated with periodic drained phases (Austin *et al.*, 2003; Stevens *et al.*, 2002). Despite this knowledge and the prediction that these organisms are widespread in a variety of environments, heterotrophs have not been considered important players in

nitrification in most environments because of lower rates of nitrification than the autotrophic ammonia oxidizers. However, since techniques such as quantitative PCR and immunofluorescent staining can now accurately enumerate AOB and typically find them to comprise just a few percent of the total population (e.g. Harms *et al.*, 2003; e.g. Urakawa *et al.*, 2006), the importance of heterotrophic nitrification could have been underestimated. Recent work, mainly using ^{15}N labelled tracers suggests that heterotrophic nitrifiers may make substantial contribution to nitrogen cycling in at least some environments, e.g. certain soils (Barraclough & Puri, 1995; Muller *et al.*, 2004; Muller *et al.*, 2006) and anaerobic riparian wetlands (Matheson *et al.*, 2003).

1.4.4 Ammonia volatization

Whilst microbial nitrification is most likely to be the predominant pathway for ammonia removal, other routes such as ammonia assimilation (Sasaki *et al.*, 2002; Sasaki *et al.*, 2004) or volatization cannot be excluded from being partly responsible in some circumstances. Volatization is only thought to be significant where ammonia is present at concentrations above 20ppm, where there is vigorous mixing, high flow rates, high pH or high temperatures (Kadlec & Knight, 1996).

1.5 Removal of nitrite by autotrophic bacteria

1.5.1 Organisms responsible for nitrite oxidation

All known nitrite oxidising bacteria belong to one of four genera: *Nitrobacter* (class α-proteobacteria, order Rhizobiales, family Bradyrhizobiaceae) has four recognised members: *N.alkalcus, N.hambergensis, N.vulgaris* and *N.winogradski*, the genera *Nitrospina* (class δ-proteobacteria , order Desulfobacteriales, family Nitrospinaceae) contains a single named species *N.gracilis*. *Nitrococcus mobilis* belongs to the γ-proteobacteria (order Chromatiales, family Chromatiaceae) and *Nitrospira* (phylum Nitrospirae; class Nitrospira, order Nitrospirales, family Nitrospiraceae) contains two named species (*N.marina* and *N.moscoviensis*) and has two candidate species *Candidatus* Nitrospira bockiana (Lebedeva *et al.*, 2008) and *Candidatus* Nitrospira defluvii (Maixner *et al.*, 2008)

There are no known nitrite oxidizing archaea, but some authors have suggested their existence is possible or even probable (Lomas & Lipschultz, 2006; Ward *et al.*, 2007).

1.5.2 Biochemistry of nitrite oxidation

Nitrite oxidation proceeds in a single step reaction using oxygen from water as an electron donor.

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \qquad (1.4)$$

The enzyme responsible for this reaction is nitrite oxidoreducatase. Oxidation probably occurs in the cytoplasm and electrons are driven through the membrane to the periplasm by the membrane potential to the more negative potential of cytochrome c. Most of the electrons pass to terminal electron acceptors (O_2), the remaining electrons are used in generation of NADH (White, 2000). *Nitrobacter* species are capable of mixotrophic growth using organic carbon sources (Koops & Pommerening-Roser, 2001).

1.5.3 Ecology of nitrite oxidizing bacteria

Nitrococcus mobilis, Nitrospina gracilis and Nitrospira marina are obligately halophilic, *Nitrobacter alkalicus* is halotolerant, all other species have no salt requirement (Koops & Pommerening-Roser, 2001). Typically wastewater will be dominated by *Nitrospira*, but *Nitrobacter* may be present under high nitrite conditions (Daims *et al.*, 2001). *Nitrospira* species also appear to be the most abundant species in marine aquaculture systems *(Foesel et al., 2008; Hovanec & DeLong, 1996; Tal et al., 2003).* One study of aquaculture waste treatment failed to detect any nitrite oxidizing bacteria using standard probes, which may indicate that there are other unidentified bacteria responsible (Paungfoo *et al.*, 2007).

1.6 Interrelation between nitrifying organisms

Co-operative relationships between different types of ammonia oxidizing bacteria have been suggested. Francis *et al* (2005) noted how several authors have observed correlations between the distribution or abundance of ammonia oxidizing archaea and anammox bacteria. It could be that under low oxygen conditions ammonia oxidizing archaea might produce both the anaerobic conditions (by consuming oxygen) and electron acceptors (nitrite) required for anammox. A similar relationship has been suggested to exist between ammonia oxidizing bacteria and anammox bacteria at aerobic-anaerobic boundaries (Schmidt *et al.*, 2002).

Studies of nitrifying biofilms typically reveal a close association between the nitrite oxidizing bacteria and ammonia oxidizing bacteria. Microscopic studies of nitrifying bacteria indicate that nitrification occurs in a narrow oxygenated surface zone of the biofilm where the ammonia oxidizing bacteria form dense aggregates around which the nitrite oxidizing bacteria cluster (Schramm *et al.*, 1996).

It is assumed that ammonia and nitrite oxidization occurs in different organisms as a result of evolutionary pressure to maximising growth rate. However it has been hypothesised that organisms with lower growth rates but increased energy yields, capable of the complete oxidation of ammonia to nitrate ("comammox") remain undiscovered (Costa *et al.*, 2006).

1.7 Microbiology of denitrification

The ability to denitrify (the reduction of nitrate to nitrous oxide or dinitrogen) is present in many bacteria and archaea (Richardson, 2000). Denitrification proceeds via multiple biochemical pathways typically under anaerobic conditions in five enzymatic steps:

$$NO_3^- \rightarrow NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$$

NAR/NAP NIR NOR NOS

Nitrate can be reduced to nitrite either by membrane bound nitrate reductase (*NAR*) or periplasmic nitrate reductase (*NAP*), then further reduced to nitric oxide by nitrite reductase (*NIR*), and to nitrous oxide by nitric oxide reductase (*NOR*) and finally to dinitrogen gas by nitrous oxide reductase (*NOS*) (Philippot, 2002).

These represent the best characterised genes for each step of denitrification, but that up to 50 genes in total might be essential for the denitrification process in a single organism (Zumft, 1997). Many autotrophic nitrifiers have the genetic capability to perform at least part of the denitrification pathway (Ye & Thomas, 2001) Ammonia oxidizing bacteria and ammonia oxidizing archaea possess both the *nir* and *nor* genes, anammox bacteria possess the *nir* gene (Francis *et al.*, 2005).

The electron donor for denitrification in organic carbon and 2.47g of methanol (or other equivalent carbon source) is necessary for the denitrification

of 1g nitrate nitrogen (Kadlec & Knight, 1996). Beside carbon limitation, elevated oxygen levels, very low or very high pH (<3.5 or >11), low temperatures or very high temperatures (>60°C) and the presence of inhibitors are known to repress denitrification (Knowles, 1982).

1.8 Constructed wetlands

1.8.1 General introduction, types of wetland

Constructed wetlands can be used to treat wastewater from wide range of sources, for example, sewage (Luederitz *et al.*, 2001), agriculture (Cronk, 1996), saline and freshwater aquaculture (Lymbery *et al.*, 2006; Schulz *et al.*, 2004), and hypereutrophic lakes (Coveney *et al.*, 2001). They are generally good for removing suspended solids, bacterial pollution and chemical and biological oxygen demand, but need careful optimisation for nitrogen and phosphorus removal (Verhoeven & Meuleman, 1999). Many factors such as the presence of wetland plants, the source and composition of wastewater, nutrient loading rate, bed composition and wetland operating parameters affect performance (Meuleman *et al.*, 2003; Sun *et al.*, 2003).

1.8.2 Comparison of waste from aquaculture and other sources

There has been some concern that wetlands might be inefficient (or uneconomical) for low concentration wastes such as aquaculture effluent

because lower nitrogen loads may kinetically limit nitrogen removal (Lin *et al.*, 2002). Wastewater from aquaculture is typically 20-50 times more dilute than municipal wastewater (Naylor *et al.*, 2003; Schulz *et al.*, 2004). One of the advantages of recirculation aquaculture is that it produces lower volumes of more concentrated effluent than flow through production systems which are more suitable for treatment in constructed wetlands (Fletcher, 2006). In the studies discussed below the highest levels of total nitrogen (TN) from aquaculture was 39.9mgl^{-1} (Lin *et al.*, 2002) though are typically much lower, e.g. 2.4 mgl^{-1} (Schulz *et al.*, 2003). In municipal waste concentrations could be 126.3mgl^{-1} (Luederitz *et al.*, 2001).

Under optimal conditions wetlands treating aquaculture waste can achieve similar removal performance to wetlands treating other wastes, under sub-optimal conditions they can perform very poorly. Table 1.1 shows nitrogen removal from aquaculture wetland studies. As a comparison, average removal from a study of 107 sewage disposal reed beds were 49% TN removal (Luederitz *et al.*, 2001). Wetlands used for aquaculture waste typically suffered from lack of denitrification attributed to a combination of short residence times and low concentrations of organic carbon (Lin *et al.*, 2005) which accounts for the low total nitrogen removal in some wetlands. However under appropriate conditions up to 98% total inorganic nitrogen (TIN) removal has been achieved (Lin *et al.*, 2002).

1.8.3 Comparison of vertical and horizontal flow constructed wetlands

Wetlands can be classified into surface- (or free water-) flow wetlands, in which the water flows above the ground surface and subsurface-flow wetlands, in which the water flows vertically or horizontally through soil or other media. Vertical flow wetlands (VFWs) are considered to be more efficient than horizontal flow wetlands (HFWs) at nitrification and removal of organic matter but show poorer denitrification (Luederitz *et al.*, 2001; Verhoeven & Meuleman, 1999). Combinations of different types can be used to maximise removal. For example in a combined HFW/VFW nitrification was observed in the VFW and denitrification in the HFW (Moir *et al.*, 2005). The use of combined systems does not always have a beneficial effect. In a subtropical wetland treating municipal wastewater the use of a VFW prior to HFW decreased the nitrogen removal when compared to the HFW alone (Bayley *et al.*, 2003). This may have been due to removal of dissolved organic carbon in the VFW, preventing denitrification in the HFW

1.8.4 Effects of hydraulic residence time and loading rates

The conventional view is that effective pollutant removal occurs with long hydraulic residence times (HRT)/low hydraulic loading rates (HLR) (Lin *et al.*, 2005) i.e. maximum contact time. For example, in a HFW treating waste from a rainbow trout farm the percentage removal of total nitrogen, nitrate and ammonia increased with longer HRT, but removal, measured in $g/m^2/d$ was highest for short residence times (Schulz *et al.*, 2003; Schulz *et al.*, 2004)

Table 1.1 summary of nitrogen removal from aquaculture wetland studies

Farmed Species	Mean Removal [1] (%)		Study
Sea bass	TAN	32.4- 65.4	(Brambilla *et al.*, 2007)
Tilapia (salinated waste)	TN	up to 98	(Brown *et al.*, 1999)
	TIN	up to 94	
Milkfish	TIN	95-98	(Lin *et al.*, 2002)
	TAN	86-98	
	NO_3^-	27.9-99	
	NO_2^-	>99	
Shrimp	TAN	57	(Lin *et al.*, 2003)
	NO_2^-	90	
	NO_3^-	68	
Shrimp	TAN	64-66	(Lin *et al.*, 2005)
	NO_2^-	83-94	
	NO_3^-	-2.4- -5.4	
Rainbow trout	TN	0-57	(Lymbery *et al.*, 2006)
Salmon (hatchery)	TAN	75.1%	(Michael, 2003)
	NO_2^-	Removed when present	
	NO_3^-	Removed when present	
Reconstituted fish farm effluent	TKN	40-90	(Naylor *et al.*, 2003)
	TAN	-287- 81.3	
	NO_3^-	44.1-69.7	
Sturgeon and Rainbow trout	TN	37	(Panella *et al.*, 1999)
	Org-N	63	
	TIN	27	
	TAN	41	
Nile tilapia	NH3	4.35-10.66	(Redding *et al.*, 1997)
	NO2	-1.06- -12.31	
	NO3	3.30-15.43	
Rainbow trout	TN	21-42	(Schulz *et al.*, 2003)
	TAN	73-91	
	NO_3^-	-31- -98	
Rainbow trout	TN	19-30	(Schulz *et al.*, 2004)
	NO_2^-	83-94	
	NO_3-	-2.4- -5.4	
Catfish	TKN	45-61	(Schwartz & Boyd, 1995)
	TAN	1-81	
	NO_3^-	43-98	
Mixed trout	TN	-1.8- 4.3	(Sindilariu *et al.*, 2007)
	TAN	49-88	
	NO_2^-	35-42	
	NO_3^-	-1.7- -1.7	
Trout	TKN	82-93	(Summerfelt *et al.*, 1999)
	NO_3^-	-570- -80000	
Shrimp	TAN	Significant decrease	(Tilley *et al.*, 2002)
	NO_3^-	No significant change	
Tilapia	TN	89	(Zachritz II *et al.*, 2008)
	TIN	95	
	NO_2^-	85	
	NO_3^-	14.5	

[1]Negative mean removal indicates an increase in the amount of measured nitrogen

suggesting that greatest removal activity in wetlands occurs in the period soon after entry to the wetland. The trend of increased nutrient removal with increased residence time is not always observed. Lin *et al.* (2002) studied the start up of a combined surface and sub-surface HFW treating milkfish (*Chanos chanos)* aquaculture waste. HLR was increased on a monthly basis from 1.8-13.5 cm/d (HRT 4.4-0.6d). Removal of ammonia, nitrite and TIN did not change significantly with HLR (TIN removal 95-98%). Effective residence time can be increased by recirculation. Recirculating water six times through a HFW treating farm effluent improved ammonium removal by over 50% (Sun *et al.*, 2003). Another study used a recirculation rate of 50-60% which improved denitrification (resulting in 72% higher TN removal) (Laber *et al.*, 1997). A significant factor in this system was the recirculation of water to the settling tank rather than the wetland inlet. The settling tank had anaerobic conditions and high organic carbon levels which encourages denitrifying bacterial activity. Addition of a carbon source (methanol) to the wetland bed also increased denitrification (Laber *et al.*, 1997). The benefit of improved nutrient removal with recirculation needs to be weighed against the operational costs of pumping (Sun *et al.*, 2003).

In addition to observing faster removal rates in the early stages of treatment, lower TN concentrations and higher nitrate plus nitrite (NOx-N) concentrations have been observed in the upper layers of a HFW (Bayley *et al.*, 2003). Reviewing other systems Cooper *et al.*, (1999) reported that the upper layer is also the most important for nitrification in VFWs and rapid infiltration

systems. Long HRT may be less important in VFWs because in some cases they are capable of nitrification even at very short residence times (measured in minutes rather than hours as in HFWs) (Moir *et al.*, 2005).

1.8.5 Wetland hydraulics

Altering the saturated and unsaturated zones can affect the performance of a wetland. This creates aerobic and anaerobic zones, maximising potential for both nitrification and denitrification. Manipulating water levels can double phosphorus and nitrogen removal in HFWs and strongly enhance removal in VFWs (Verhoeven & Meuleman, 1999), although including a saturated zone may decrease nitrification by reducing the available aerobic volume (Laber *et al.*, 1997). An experimental tidal flow system which was operated on a four hour cycle, short (one hour) saturated and long (three hour) unsaturated conditions produced the best nitrogen removal and was the only condition to display evidence of denitrification (Zhao *et al.*, 2004). In these wetlands more NH_3-N was removed than could be accounted for by NO_2-N and NO_3-N production, this combined with pH increases led to authors to conclude that adsorption, biomass assimilation and plant uptake were more important than nitrification in this wetland. An experimental system that periodically emptied and refilled gravel beds with wastewater showed that six fluctuations per day increased ammonia removal in both planted and unplanted cells (99% and 92% removal respectively compared to 50% and 17% in static cells), but accumulation of NO_x-N was observed, limiting total nitrogen removal to 60% (planted) and 55% (unplanted)

compared to 50% and 17% in static cells (Tanner *et al.*, 1999). Laber *et al.* (1997) observed that nutrient removal from wastewater loads applied four times a day was more effective than a single daily application (although the authors conceded that this might be explained by lower average temperatures in the second experimental period). Applying waste water in batches has the benefit that storing water prior to release allows more even distribution over the wetland surface (Laber *et al.*, 1997). As well as batch loading, wetlands are typically operated with a resting period which allows decomposition of accumulated organic material and aeration of soil based beds allowing nitrification to occur (Metcalf & Eddy, 1991).

1.8.6 Vegetation effects

Planting wetlands can be an effective way of increasing nutrient removal. de Sousa *et al.* (2003) found that the removal efficiency of total Kjeldahl nitrogen (TKN) was up to 56% better in wetlands planted with *Juncus* spp compared with identical unplanted cells. Improved nitrogen removal has also been observed in planted lab scale wetlands loaded with primary treated wastewater (Lim *et al.*, 2001) and freshwater fish farm effluent (Naylor *et al.*, 2003). Plant uptake was estimated to account for 50% nitrogen removal in a VFW (Meuleman *et al.*, 2003). Nitrogen removal in a wetland may change according to the cyclic growth of wetland macrophytes, and may be lower with mature plants than with plants in growth phase (de Sousa *et al.*, 2003).

1.8.7 Temperature effects

Several authors have noted that ammonia removal increases with temperature. (Sun *et al.*, 2003) observed a trend of increased removal over the range of 0 -15°C. Lin *et al.* (2005) reported increased removal in the warm season. These increases are not necessarily due to increased microbial activity, Morris and Herbert (1997) concluded that elevated temperature (27°C) of waste from a sugar beet processing system improved ammonia removal through increased volatisation rather than temperature enhanced nitrification

1.8.8 Summary of practical application of above data on nitrogen removal

The optimisation of wetlands to remove nutrients is a complex process, with variables associated with the wetland and the wastewater needing to be considered in each case. Providing conditions that allow both nitrification and denitrification is crucial to efficient nitrogen removal and the use of combined HFW/VFWs seems to be the best solution. Reviewing sewage treatment wetlands in Germany, Luederitz *et al.* (2001) recommended using combined HFW/VFW systems, intermittent loading, long HFW distances and a naturally aerated pond pre-treatment (particularly if using only VFWs with limited denitrification) for maximum nitrogen removal. Two sequences of combined bed have been suggested: either a VFW to remove BOD, COD, bacteria and carry out nitrification followed by a denitrifying HFW (an additional small HFW could be added prior to VFW to remove suspended solids and prevent clogging of the VFW), or a HFW followed by a VFW with effluent being recirculated to the HFW

inlet so that denitrifying bacteria can utilize the higher carbon levels (Cooper, 1999; Cooper *et al.*, 1999). Another solution could be to create aerobic and anaerobic zones in the same bed by fluctuating the water level (Tanner *et al.*, 1999; Zhao *et al.*, 2004) and the use of rest periods or intermittent loading (Laber *et al.*, 1997). If carefully optimised, such an approach should allow nitrification and denitrification in the same wetland.

1.9 Nitrogen removal in wetlands treating aquaculture waste

The majority of data on the treatment of aquaculture waste in wetlands comes from laboratory or pilot scale wetlands. Aquaculture waste is considered difficult to treat because of the low concentration and high volumes of waste. When compared to treatment in a settlement basin, constructed wetlands were found to be more efficient at nitrogen removal, mainly through achieving ammonia and nitrite removal, rather than production as was seen in the settlement basins (Sindilariu *et al.*, 2007). The problems of low nutrient concentrations were highlighted in a study which showed that surface flow wetlands showed a significant removal of ammonia and total nitrogen during a farm cleaning situation with higher nutrient loading periods (80.5gm^{-2} TN) but not under normal operating conditions (54.2gm^{-2} TN) (Sindilariu *et al.*, 2007). Similarly a subsurface HFW removed up to 50% more TN (69%TN removal) at high nutrient concentrations (2.97-3.77 mgl^{-1} TN) than at low concentrations (0.82-0.73 mgl^{-1} TN) (Tilley *et al.*, 2002).

Limited nitrate removal and poor ammonia removal or ammonia production appear to be particular problems associated with aquaculture wastewater treatment. Increases in ammonia have been seen in several studies. Summerfelt (1999) observed ammonia increases in both horizontal and vertical flow wetlands treating effluent from a trout (*Oncorhynchus mykiss*) farm. A surface flow HFW treating mesohaline Pacific white shrimp (*Liptopenaeus vannamei*) aquaculture waste, operating with a HRT of 24h saw no nitrification occurring in the wetland. Ammonia concentrations rose in the wetland, but always remained below 1.8mg/l. (Tilley *et al.*, 2002). In both cases they concluded that the ammonia increases were due to mineralisation of organic nitrogen, combined with insufficient nitrification due to oxygen limitation (because of high sediment oxygen demand). Another wetland treating waste from a shrimp farm that used a combined surface water wetland and subsurface wetland with a shorter HRT (2.3-1.6h) was more successful at decreasing ammonia by 66/64% in the Taiwanese warm and cold seasons respectively (Lin *et al.*, 2005)

Another frequent problem in aquaculture wetlands is that efficient removal of nitrates is difficult to achieve. Poor nitrate removal may been seen at the same time or independently of ammonia production. No significant removal of nitrate was seen by Sindilariu *et al* (2007) and Tilley (2002) in either vertical flow or horizontal flow wetlands. Nitrate increases have been observed in both horizontal and vertical flow wetlands, despite similar levels of TKN removal in both types (89% VF, 86% HF). The nitrate increases were smaller in horizontal

flow wetlands (Summerfelt *et al.*, 1999). Reviewing previous data Lin *et al.* (2005) observed that good nitrate removal (68-99%) was seen in systems with low HLRs (<0.3m/d), hence longer HRTs (Lin *et al.*, 2002; Lin *et al.*, 2003). In similar systems with higher HLRs (>1.03m/d) (Lin *et al.*, 2005; Schulz *et al.*, 2003; Schulz *et al.*, 2004) nitrate removal was poor or nitrate increased. In both Schulz *et al.* studies nitrate removal efficiency was negatively correlated with HRT, and no significant removal of nitrates was seen with HRTs up to 11 hours. Curiously nitrate removal dropped from 99% to less than 82.4% in one study for HRTs longer than 6.8d (Lin *et al.*, 2002) (although it should be noted that this change in nitrate removal coincided with the beginning of total nitrogen removal in the surface flow cell in this start up experiment. Therefore it could reflect increased nitrification rather than a direct effect of reduced denitrification caused by longer HRT).

One further study of interest looked at using aquaculture wastewater to irrigate salt tolerant plants (Brown *et al.*, 1999). Although not set up as a treatment wetland there were two interesting findings that may be relevant to the current study. Firstly, increasing the salinity to 35ppt (the typical concentration of seawater) inhibited removal of total nitrogen and inorganic nitrogen. This is in contrast to a study by Lymberry *et al.,* (2006), which found high salinity (>24ppt NaCl) did not reduce TN removal when compared with low salinity (<7.5ppt NaCl). The second important point to come from this study is that removal of inorganic nitrogen from planted lysimeters was much greater than in unplanted lysimeters (94% compared to 58%), but the difference was much

smaller for total nitrogen (98% compared to 96%). This supports the data from wetland studies that show that planting a wetland only makes small improvements to total nitrogen removal, but does indicate that plants have a more important role in the removal of the inorganic nitrogen component of wastewater.

1.10 The importance of developing treatment technologies for the aquaculture industry and the scope of this study

Aquaculture is a rapidly expanding industry, currently producing almost half of the 96.3 million tonnes of aquatic food consumed each year (FAO, 2004; FAO, 2006). It therefore has an important role in reducing the pressure on wild fisheries by producing fish for consumption using sustainable methods. However, as with any industry there is a need to minimise the environmental impacts that inevitably result. Sustainable aquaculture protects not only the environment, but also the long term interests of fish producers and improves consumer confidence through promotion of environmentally friendly standards (Boyd, 2003). In order to address these concerns the aquaculture industry needs to promote sustainable practices relating to the acquisition of stock and feedstuffs, biological interactions outside the aquaculture facility, habitat change and treatment of waste products (Royal-Commission-on-Environmental-Pollution, 2004). Inland marine fish farming addresses some of these concerns

through the physical separation of wild and farmed fish, thus reducing biological interaction and by allowing waste to be handled in a more effective way, such as by treatment in constructed wetlands.

While there is a substantial body of work on the efficacy and microbial processes of freshwater wetlands, there is little comparative information for saline wetlands. In particular due to the relative novelty of land-based marine fish farms, information for saline treatment wetlands as used in the treatment of aquaculture waste is limited. It is important to determine the specific characteristics of saline aquaculture bioremediation wetlands. There is some disagreement in the literature whether or not salinity affects nitrogen removal but there are several reasons why nitrification in particular might be lower in saline aquaculture wetlands. Firstly, adsorption of ions to charged sites on surfaces is lower in saline water, which may reduce the availability of ions to biofilm bacteria responsible for their removal (Rysgaard *et al.*, 1999; Seitzinger *et al.*, 1991). Secondly, studies of ammonia oxidizing bacteria from along estuarine salinity gradients have shown that potential nitrification rates are reduced at salinities above around 10ppm even for bacteria isolated from more saline environments (Bernhard *et al.*, 2007; Magalhaes *et al.*, 2005). Thirdly, an equilibrium exists between adsorbed and free ions, therefore the amount of ammonia adsorbed to the biofilm and therefore available for nitrification will be low where ammonia concentration is relatively low (Vymazal, 2006), as is typical for aquaculture effluents. These aspects of nitrification capacity further highlight the difficulties that need to be taken into account when designing constructed bioremediation

wetlands for marine aquaculture farms.

This study investigated the use of constructed wetlands for the treatment of aquaculture wastewater. The field site was a newly constructed wetland at the Selonda UK land-based marine fish farm on Anglesey, north Wales. The wetland had been designed specifically to treat wastewater from this new 1200 tonnes per annum facility. The purpose of this study was to gain a greater understanding of the processes involved in nitrogen removal in these wetlands. The aims were:

- to use molecular techniques to monitor the total bacterial and ammonia oxidizing bacterial communities in the Selonda UK wetland during the first 17 months of its operation
- to use laboratory-scale models to investigate the ability of wetlands to treat aquaculture wastewater that contained high concentrations of ammonia
- to use model wetlands to compare the performance of flood/drain and submerged wetlands
- to investigate changes in the total bacterial and ammonia oxidizing communities in both models
- to use the data from the models to make recommendations for operation of the Selonda UK wetland.
- to carry out a study at the Selonda UK wetland, based on those recommendations, to discover whether the results from the model systems could "scaled up" to full size wetlands.

Chapter Two

Materials and Methods

2.1 Chemicals and media

All commonly used media and chemicals are shown in tables 2.1 and 2.2 respectively. Unless otherwise indicated solutions were autoclaved at 121°C, 15psi for 15 minutes. De-ionised water (dH_2O) was obtained from a Milli-Q water purification system.

Table 2.1. Growth media used

Medium	**Ingredients**	**Quantity per litre dH_2O (except where indicated*)**
Luria Bertani (LB) Broth and agar	Tryptone Yeast extract NaCl dH_2O for plates add 10g agar	10g 5g 5g Up to 1L
SOC	Tryptone Yeast extract 1M NaCl 1MKCl H_2O after autoclaving add 1M $MgCl_2.6H_2O$ 1M $MgSO_4.7H_2O$ 1M glucose	20g 5g 10ml 2.5ml Up to1L 10ml 10ml 10ml

Table 2.2. Commonly used reagents

Solution	Ingredients	Quantity per litre dH_2O (except where indicated*)
Alkaline citrate	Trisodium citrate Sodium hydroxide	200g 10g
*Ampicillin	Ampicillin dH_2O filter through a 0.22μm filter, store at -20°C	50mg Up to 1ml
*Bromophenol blue DNA loading dye	Sucrose Bromophenol blue dH_2O	40g 0.25g 100ml
*IPTG (Isopropylthio-β-D-galactoside -	IPTG dH_2O Do not autoclave, filter through 0.22μm filter. Store at -20°C	2g up to 10ml
Kjeldahl Digestion solution	K_2SO_4 $CuSO_4$ dH_2O conc. H_2SO_4 add while swirling, cool H_2O	134g 7.3g 800ml then 134ml up to 1L
*Oxidizing solution	Alkaline citrate (see above) 5% sodium hypochlorite solution	100ml 25ml
*Phenol solution	Phenol (>89% purity) 95% v/v ethanol	11.1ml 98.9ml
10x TBE (Tris Borate EDTA)	Tris Boric acid EDTA	108g 55g 9.3g
TE (Tris EDTA)	Tris EDTA pH8.0	1.21g 372mg
*X-gal (5-Bromo-4chloro-3-indolyl)	X-gal Dimethylformamide Do not autoclave or filter, store at -20°C	2g up to 10ml

2.2 Preparation of samples for T-RFLP analysis

2.2.1 Sample collection

Biofilm samples were collected from the limestone gravel in the wetlands (model and full scale) as follows. Pieces of gravel were removed from the wetland, from approximately 5cm beneath the surface. Using a sterile scalpel the surface was scraped until approximately 0.1 – 1.0g material was collected. For microbial analysis of water samples at the Selonda UK wetland, 500ml water samples were collected in sterile containers. All samples from the field site were stored on ice for transport to the laboratory. Samples were stored at -20°C until DNA was extracted. Immediately prior to storage at -20°C, water samples were centrifuged at 4500rpm for 10 minutes to pellet the suspended cells, and supernatant was removed.

2.2.2 DNA extraction

DNA was extracted using a fastDNA SPIN kit for soil (Q-Biogene, Cambridge, UK) as follows. Approximately 0.5g of the collected biofilm sample was added to a lysing matrix tube (containing ceramic and silica particles, designed to improve lysis of microorganisms). Alkaline lysis was carried out by addition of 980µl of sodium phosphate buffer and 120µl MT buffer to the tube. For effective lysis tubes were placed in a FastPrep® Instrument and vigorously shaken at speed 5 for 30 seconds. The lysing

matrix tubes were then centrifuged for five minutes at 13000rpm to pellet unwanted cell debris. Protein was precipitated by transferring the supernatant to a clean tube, adding 250µl of PPS (protein precipitation solution) and inverting the tubes ten times by hand. The tubes were then centrifuged at 13000rpm for 1 minute and the supernatant was transferred to a 15ml tube containing 1ml binding matrix suspension. After gently mixing the tubes for 2 minutes to allow binding of DNA to the matrix, the tubes were allowed to stand in a rack for 3 minutes to allow settling of the silica matrix. 500µl supernatant was removed and discarded. The remaining binding matrix was resuspended and transferred to a SPIN™filter, centrifuged for 1 minute at 13000rpm to capture the matrix-bound DNA. The DNA was washed by a further 1 minute spin at 13000rpm with 500µl SEWS-M. An additional centrifugation removed residual SEWS-M and the DNA was eluted into a fresh catch tube by adding 50µl dH_2O and centrifuging for 1 minute at 13000rpm. Samples were stored at -20°C. DNA quantifications were carried out using a NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, Oxfordshire, UK).

2.2.3 Polymerase chain reaction

PuReTaq Ready-to-go beads (Amersham Biosciences, Buckinghamshire, UK) were used for PCR reactions. 0.5µl DNA was added to a PCR mix containing 2.5 units puReTaq DNA polymerase, 200µM each dNTP, 0.2µM each primer, 1.5mM $MgCl_2$, 50mM KCl in 10mM Tris-HCl. The

forward primer for T-RFLP reactions was labeled with a Beckman D3 dye (Proligo France SAS, Paris, France). PCRs were carried out on a PTC-200 DNA Engine (M.J.Research Inc. Waltham MA, USA) in triplicate and the three PCR products were pooled before analysis to reduce PCR amplification bias. A no template (negative control) reaction was always carried out.

Table 2.3. Universal eubacterial primers targeting 16S rRNA and *amoA* genes.

gene	Primer name	sequence	reference
16S rRNA	WellRED D3 labelled 27f	5'[D3]-AGA GTT TGA TCM TGG CTC AG-3'	(Lane, 1991)
16S rRNA	1387r	5'-GGG CGG WGT GTA CAA GGC-3'	(Marchesi *et al.*, 1998)
amoA	*amoA*-1F[1]	5'-GGG GTT TCT ACT GGT GGT-3'	(Rotthauwe *et al.*, 1997)
amoA	*amoA*2R-TC	5'-CCC CTC TGC AAA GCC TTC TTC-3'	(Nicolaisen & Ramsing, 2002)

[1]An unlabelled primer of the same sequence was used when the PCR product was intended for cloning and sequencing

16s rRNA PCR program:

Initial denaturation: 95°C for 5 minutes followed by 30 cycles of:

Denaturation : 95°C for 30 seconds

Annealing: 66°C for 45 seconds

Extension: 72°C for 75 seconds

Followed by a final extension step of 72°C for 10 minutes.

***amoA* program:**

Initial denaturation: 95ºC for 2 minutes followed by 35 cycles of:

Denaturation : 95ºC for 30 seconds

Annealing: 55.5ºC for 45 seconds

Extension: 72ºC for 60 seconds

Followed by a final extension step of 72ºC for 10 minutes.

2.2.4 Agarose gel electrophoresis

PCR reactions were checked visually by electrophoresis. 0.8% w/v gels were used to visualize the 16s rRNA and *amoA* PCR products (and plasmid digests). 50 or 100ml gels were produced by addition of the appropriate weight of agarose to 1xTBE buffer with 0.5µgml^{-1} ethidium bromide. 3µl PCR buffer was mixed with 1µl bromophenol blue loading dye and run for 30 minutes at 100V. DNA was visualized using a Bio-Rad UV transilluminator (Bio-Rad, Hemel Hempstead, UK) at 245nm.

2.2.5 Restriction endonuclease digestions

Typically enzyme digests were carried out as follows

xµl DNA

3µl digestion buffer

1µl enzyme

26 - xµl dH_2O

For T-RFLP 12.5µl 16s rRNA PCR product was digested with *Alu*I in buffer 2 and 7.5 µl *amoA* PCR product digested with *Hph*I in buffer 4. When preparing fragments for sequencing, inserts were released from the plasmid using *Eco*RI. All digests were carried out for 5 hours or overnight at 37°C.

2.2.6 PCR purification

Excess PCR primers were removed using QIAquick PCR purification kit (Qiagen, Crawley, UK). PCR samples were mixed with 5 times the volume of Qiagen's binding buffer PB. This mixture was added to a QIAquick spin column and the DNA was then bound to the silica membrane by centrifuging for 1 minute at 13000rpm. The flow-through was discarded and the DNA washed by centrifugation for 30 seconds at 13000rpm with 750µl Qiagen's wash buffer PE. Flow-through was discarded and the spin column was centrifuged for 2 minutes to remove excess wash buffer. DNA was eluted by adding 30µl DNase/pyrogen free water to the centre of the membrane, the column was allowed to stand for 1 minute before collecting the sample in a 1.5ml Eppendorf tube by centrifugation.

2.2.7 Fragment analysis

3µl was mixed with 37 µl sample loading solution (Beckman Coulter, High Wickham, UK) and fragment analysis was carried out using Beckman CEQ 8000 capillary electrophoresis, using either a 1000bp custom map marker ladder or a 640bp standard (Beckman Coulter, High Wickham, UK). Samples were with a capillary temperature of 50°C on the following program:

Denature:	120 seconds
Injection:	2kV for 45s
1° separation:	5kV ramp duration 2 minutes
2° separation:	5kV, start time 10 minutes, ramp duration 5 minutes

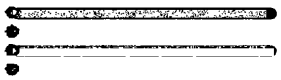
Total separation time: 75 minutes

To minimize false reporting of background noise the cut-off level for peak recognition was kept at the default of 10%. As this kind of analysis is known to produce "shoulder peaks" around the main product the *amoA* T-RFLP profiles were edited by eye. However because many of the peaks in the 16s rDNA profiles were so close together these shoulder peaks could not be confidently discriminated from actual peaks so this data was left unedited. T-Align (Smith *et al.*, 2005) was used to identify common fragments. After using the moving average function of this software the resulting grouping of fragments was edited by eye to reduce mis-grouping of peaks. Multivariate statistical package (MVSP) (Kovach computing services, Anglesey, Wales) was used to carry out principle component analysis on the T-RFLP data. In order to avoid the over representation of changes in rare species and to make the data suitable for linear analysis by PCA, the relative abundance data obtained from the T-RFLP was transformed using a chord transformation (Ramette, 2007). The scores of the first two principal components were used to compare differences between the T-RFLP profiles.

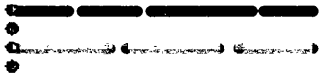
Terminal Restriction Fragment Length Polymorphism Analysis (T-RFLP)

1. Collect biofilm and extract DNA

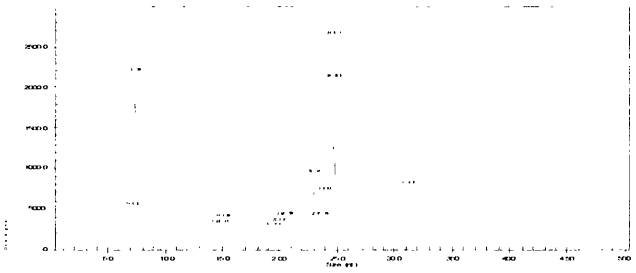
2. Amplify DNA using group specific fluorescent labelled PCR primers: Produces a PCR product containing DNA from all species in sample (represented below by different colours)



3. Digest with restriction enzyme: Produces terminal restriction fragments of different length



4. Separate and detect terminal restriction fragments (TRFs) by capillary electrophoresis



5. Convert data to 3D relative abundance graphs and display several samples on one set of axes

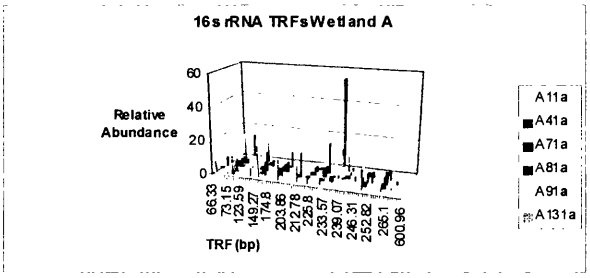


Figure 2.1. Schematic showing the steps from environmental sample to graphical display of T-RFLP data

Since each terminal restriction fragment (TRF) usually represents several species, the phrase operational taxonomic unit (OTU) is used to describe the organisms represented by a particular peak.

2.3 Cloning of PCR products

2.3.1 Vector ligation

For sequencing of *amoA* PCR products and PCR products (amplified with unlabelled primers) were cloned into pGEM®-T Easy vector (Promega UK, Southampton, UK). The following components were mixed and incubated overnight at 4°C.

5µl 2X rapid ligation buffer

1µl pGEM-T Easy vector (50ng)

2µl Purified PCR product

1µl T4 DNA ligase (3 Weiss units/µl)

1µl dH_2O

2.3.2 Transformation of electrocompetent E.coli JM109

1µl ligated vector was added to 40µl electrocompetent JM109 cells (stored at -70°C and thawed on ice). After a 2 minute incubation on ice the mixture was transferred to a chilled electroporation cuvette and electroporated using program EC1 on a MicroPulser (Bio-Rad, Hemel Hempstead, UK). 700µl SOC medium was added and the mixture was

incubated for 90 minutes in a shaking incubator at 225rpm for 90 minutes at 37°C. The mixture was plated onto LB agar with ampicillin (50μg/ml), to select for the pGEM®-T Easy vector. X-gal (25μg/ml) and IPTG (25μg/ml) were added to allow blue white screening for the presence of the insert. Plates were incubated overnight at 37°C.

2.3.3 Plasmid isolation

White colonies (indicating the disruption of the *lacZ* gene by an inserted piece of DNA) were picked and grown in LB broth (with 50μg/ml ampicillin) overnight, and plasmids were recovered using a SV miniprep wizard plus kit (Promega UK, Southampton, UK). Cells were pelleted by centrifugation at 4500rpm for 10 min. 250μl Promega resuspension solution (50mM Tris-HCl (pH 7.5,) 10mM EDTA, 100μg/ml RNase A) was added and the cells were resuspended by gentle pipetting. 250μl Promega lysis solution (0.2M NaOH, 1% SDS) and 10μl alkaline protease solution were added (with gentle mixing by inversion after addition of each. Samples were incubation for 5 minutes at room temperature then neutralised with 350μl neutralisation solution (4.09M guanidine, 0.759M potassium acetate, 2.12M glacial acetic acid), centrifuged for 5 minutes at 13000rpm and the cell lysate was transferred to a spin column. DNA was captured in the DNA binding matrix by centrifugation at 13000rpm for 1 minute. After two washes with 750μl and then 250μl Promega wash solution (60% ethanol, 60mM potassium acetate, 8.3mM Tris HCl (pH 7.5), 0.04mM EDTA) (centrifuged 1 minute at 13000rpm) the matrix was dried by a further centrifugation at 13000rpm for 2 minutes. DNA was eluted by a 1 minute 13000rpm centrifugation with 50μl dH_2O.

Confirmation of the presence of the correct insert was confirmed by enzymatic digest with *Eco*RI and agarose gel electrophoresis.

2.3.4 Sequencing and alignment

For sequencing 2µl plasmid DNA and 8µl dH_2O were mixed together in a 0.2µl tube and heated at 95°C for 2 minutes. 8µl DTCS and 2µl primer was added to the tube and placed in the thermal cycler for thirty cycles of 96°C for 20s followed by 60°C for 4 minutes. The following standard sequencing primers were used to sequence inserts.

1224 5'-CGCCAGGGTTTTCCCAGTCACGAC-'3

1233 5'-AGCGGATAACAATTTCACACAGGA-'3

Sequencing was carried out using a Beckman CEQ8000 (Beckman Coulter, High Wickham, UK)

2.3.5 Phylogenetic analysis of amoA clones and database sequences

92 *amoA* sequences selected from GenBank were aligned with 19 sequenced *amoA* clones from this study. A neighbour joining tree with the Kimura two-parameter substitution model, complete elimination of incomplete data and bootstrapping of 1000 resamplings was constructed using MEGA version 4.0 (Tamura *et al.*, 2007) . The alignment was then subjected to *in silico* restriction enzyme digest (http://insilico.ehu.es/restriction/prealign_seq/)

with multiple enzymes to determine which enzyme would best discriminate between different phylogenetic groups (Bikandi *et al.*, 2004).

2.4 Water chemistry

2.4.1 Ammonia concentration determination N-NH_3 (Hach Salicylate method 10023)

This test was used to determine ammonia concentration in the ammonia loading experiment (Chapter 3).

2ml water sample (or sample diluted with dH_2O to achieve a concentration within the 0.02 to 2.50 mg/L NH_3–N range of the test) was added to one AmVer™ Diluent Reagent Test'N Tube (Hach Lange, Salford, UK). One ammonia salicylate reagent powder pillow was added to the tube followed by one ammonia cyanurate reagent powder pillow. The tube was capped and shaken until the powder was dissolved. Ammonia compounds in the sample react with chlorine to form monochloroamine. This reacts with the salicylate to form 5-aminosalicylate in the presence of sodium nitroprusside to form a blue coloured compound (the solution appears green due to masking by the yellow colour of excess reagent). The intensity of this colour is proportional to the concentration of ammonia. After a 20 minute reaction time the concentration was measured using a Hach DR/2500 spectrophotometer

(Hach Lange, Salford, UK). Test results were measured at a wavelength of 655nm. The precision and sensitivity of this test is shown in table 2.3.

Table 2.3 Precision and sensitivity of Hach tests (data taken from procedures manuals)

Test	**Precision (95% confidence limits of distribution unless stated otherwise)**	**Sensitivity (change in concentration for a 0.010Abs change in absorbance)**
NH_3-N: DR/2500 method10023	±4% using 1mg/L NH_3-N standard	Over entire range: 0.015mg/L NH_3-N
NO_2-N: DR/2500 method 10019	±4% using 0.150mg/L NO_2-N standard	Over entire range: 0.003mg/L NO_2-N
NO_3-N: DR/2500 method 8039	±8% using 10mg/L NO_2-N standard	At 0mg/L: 0.5mg/L NO_3-N At 10mg/L: 0.7mg/L NO_3-N At 30mg/L: 0.8mg/L NO_3-N
NH_3-N: DR/700	SD=±1.5% using 0.400mg/L NH_3-N standard	Data not provided

2.4.2 Nitrite concentration determination N-NO_2 (Hach Diazotization method 10019)

The Hach test for nitrite was used in the ammonia loading experiment (Chapter 3) and the flood/drain model experiments (Chapter 4).

5ml water sample (or sample diluted with dH_2O to achieve a concentration within the 0.003 to 0.500 mg/L NO_2–N range of the test) was added to a Test'N'Tube NitriVer3 vial (Hach Lange, Salford, UK) and shaken to dissolve the powder. During a 20 minute reaction time the nitrite reacted with sulphanilic acid, which couples with chromotropic acid to produce a pink solution. The intensity of this colour was proportional to the concentration of nitrite. Samples were analysed using a Hach DR/2500 spectrophotometer

(Hach Lange, Salford, UK). Test results were measured at a wavelength of 507nm. The precision and sensitivity of this test is shown in table 2.3.

2.4.3 Nitrate concentration N-NO_3 (Hach Cadmium reduction method 8039)

The Hach test for nitrate was used in the ammonia loading experiment and the flood/drain model experiments (Chapter 4).

One NitraVer5 AccuVac ampule (Hach Lange, Salford, UK) was immersed in 50ml water sample (or sample diluted with dH_2O to achieve a concentration within the 0.3 to 30.0 mg/L NO_3–N range of the test). The tip of the ampule was snapped off against the edge of the container. The tip was kept immersed while the ampule filled. Samples were inverted 48-52 times in one minute, then left for five minutes for the reaction to take place. Cadmium in the NitraVer5 ampule reduced the nitrates to nitrites. Nitrite ions react with sulfanilic acid to form an intermediate diazonium salt. When this reacts with gentisic acid an amber colour solution forms, the intensity of which is proportional to the concentration of nitrate. Samples were analysed using a Hach DR/2500 spectrophotometer (Hach Lange, Salford, UK). Test results were measured at a wavelength of 430nm. This test gives concentration of nitrate plus nitrite (due to the reduction of nitrates by cadmium during the test). To obtain nitrate concentration, nitrite concentration obtained by the method described in 2.4.2 was subtracted from this value. The precision and sensitivity of this test is shown in table 2.3.

2.4.4 Total Kjeldahl nitrogen and ammonia determination by standard methods

These tests were used for the flood/drain experiment (Chapter4).

To measure total Kjeldahl nitrogen, amino nitrogen in organic matter was converted to ammonia in the presence of sulphuric acid, potassium sulphate and cupric sulphate. Then ammonia concentration was measured in the samples. Organic nitrogen was obtained by subtracting the ammonia concentration from the TKN in each sample. 1ml digestion solution was added to 2.5ml water sample (or sample diluted with dH_2O to achieve a concentration within the range of the test) in a 25ml boiling tube. The tube was covered with a loose top, weighed and placed in a block digestor at 185°C for three hours. Tubes were allowed to cool and were reweighed (an adjustment was made in the concentration calculation for evaporative loss during heating). 200µl sample was mixed with 2.3 ml alkaline citrate

Ammonia determination: 2.5ml water sample (water sample or digested TKN sample) was added to a 10ml test tube. 0.1ml phenol solution, 0.1ml sodium nitroprusside (0.5%w/v) solution and 0.25ml oxidizing solution were sequentially added to the sample with mixing of the tubes after the addition of each. A bung was added to the test tube while the colour reaction (caused by the reaction of ammonia with phenol and alkaline hypochlorite to give indophenol blue) was allowed to proceed at room temperature and in subdued light for one hour. The nitroprusside intensifies the blue colour. Absorbance was measured at 640nm and concentration was calculated by comparison to a standard curve calculated over the range of 0.1 to 1.0mg/L NH_3-N.

2.4.5 Field sampling of N-NH_3 (Salicylate method)

This test was used for the sampling at the fish farm wetland (Chapters 5 and 6).

5ml water sample was added to a reaction tube and the contents of one Hach ammonia salicylate reagent powder pillow was added and mixed thoroughly. After two minutes the contents of one Hach ammonia cyanurate powder pillow was added to the tube. The sample was mixed thoroughly until the powder dissolved and the colour reaction was allowed to proceed for 15 minutes. (The principle of this colour change is described in section 2.4.1). In addition to the samples, a blank containing distilled water and reagents was prepared. Concentration of ammonia was measured using a portable Hach colorimeter model DR/700 at 610nm (Hach Lange, Salford, UK). Precision of this test is shown in table 2.3.

2.4.6 Field sampling of N-NO_2 (Diazotization method)

This test was used for the sampling at the fish farm wetland (Chapters 5 and 6).

The contents of one sachet of Hach NitriVer5 reagent powder pillow (Hach Lange, Salford, UK) was added to 5ml water sample in a reaction tube. The tube was shaken for 1 minute, and then left for 10 minutes while the colour reaction proceeded (as described in section 2.4.2). Concentration was measured using a Hach colour comparator, Model NI-12 (Hach Lange, Salford, UK) using a sample of the untreated water as a blank.

2.4.7 Field sampling of N-NO_3. (Cadmium reduction method)

This test was used for the sampling at the fish farm wetland (Ch 5 and 6).

The contents of one sachet of nitraver5 reagent powder pillow (Hach Lange, Salford, UK) was added to 5ml water sample, shaken for 1 minute, then left for 1 minute while the colour reaction occurred (as described in section 2.4.3). Concentration was measured using a Hach colour comparator, Model NI-12 (Hach Lange, Salford, UK) using a sample of the untreated water as a blank.

2.4.8 Dissolved oxygen

Dissolved oxygen was measured using an Oxyguard Handy Polaris waterproof dissolved oxygen & temperature meter (Sterner Aqua Tech, Inverness, UK).

2.4.9 Statistical analysis of water chemistry analysis flood/drain wetlands

Statistical analysis was carried out using SPSS version 13.0 (SPSS Inc., Chicago, USA). A Kolmogorov-Smirnov test for normality was applied to the water chemistry data. The data was not found to conform to normality therefore non parametric analysis was used to compare different conditions. As a measure of the performance of each of the conditions, Mann-Whitney U tests were used to compare the outlet concentration in the flood/drain condition with the submerged condition. To identify whether the outlet concentration was significantly different to inlet concentration, either Wilcoxon signed-rank tests or Mann Whitney U tests were performed. In the model wetlands the same batch of water was tested at the inlet and outlet of the wetland so paired analysis of means was carried out (i.e. Wilcoxon

signed-rank), for the full scale wetlands this batch mode of operation was not applicable so unpaired analysis was used (Mann Whitney U tests). Pearson's coefficient correlation (r) was calculated as a measure of the effect size.

2.5 Model and field site wetland descriptions

2.5.1 Vertical trickle flow model wetland design and operation

Three replicate model wetlands were constructed as in Figure 2.2. For each model, five perforated crates (600 X 400 x 200mm) were packed with three grades of gravel (upper crate 0-30mm diameter gravel, second and third crates 30-80mm diameter and lower two crates 80-150mm diameter). The overall height of the wetlands was approximately the same as the depth of the full scale wetland (1.2m). Aquaculture wastewater was continuously circulated through the wetland at a rate of approximately 5L/min (100% recirculation in approximately 24 minutes) using a perforated tray to ensure even distribution of water over the surface of the wetland. During the experiment, water temperature was between 24.5ºC and 28ºC and salinity between 28ppt and 38ppt. Each of the wetlands was dosed weekly with effluent from a commercial marine fish farm. The effluent used was the supernatant collected following settlement of solids prior to release into the wetland. At the start of the experiment 60L effluent was added to the wetland. Water samples were taken at this point (inlet) and after seven days treatment (outlet). Immediately after taking the outlet sample, 25L was

removed from the each of the sump tanks and 25L fresh effluent was added. The water in the wetland was supplemented by 5.25g ammonium chloride. This process was repeated each week except that the amount of ammonium chloride was doubled each week until a reduction in the ammonia removal rate was observed. After this occurred, monitoring continued for a further four weeks. During this period ammonium chloride was added to try to

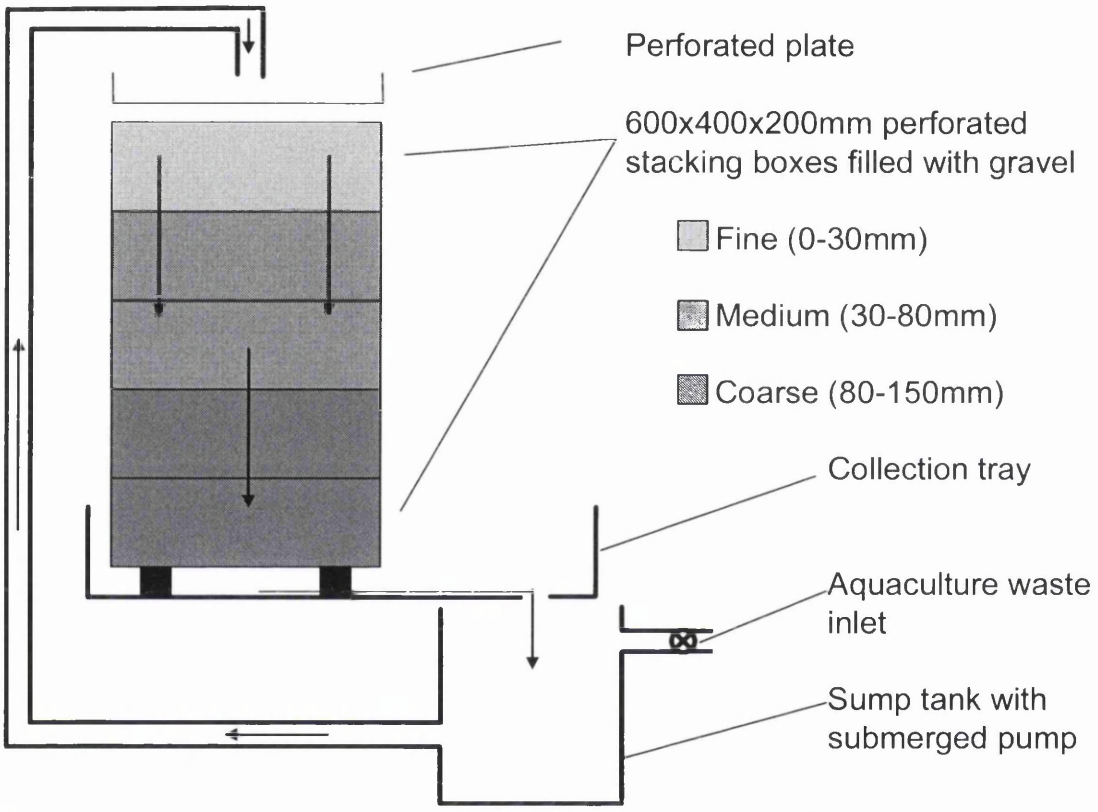


Figure 2.2. Diagram of cross section through one of the three replicate wetland models. Arrows indicate direction of water flow. Wetlands were covered in plastic sheeting during operation to minimize loss of water.

maintain the inlet concentration at around 1000ppm N- NH_3. Since the addition of the new wastewater occurred prior to analysis of the water from the previous week, the amount of ammonium chloride required to achieve a final concentration of 1000ppm had to be estimated. Actual concentrations

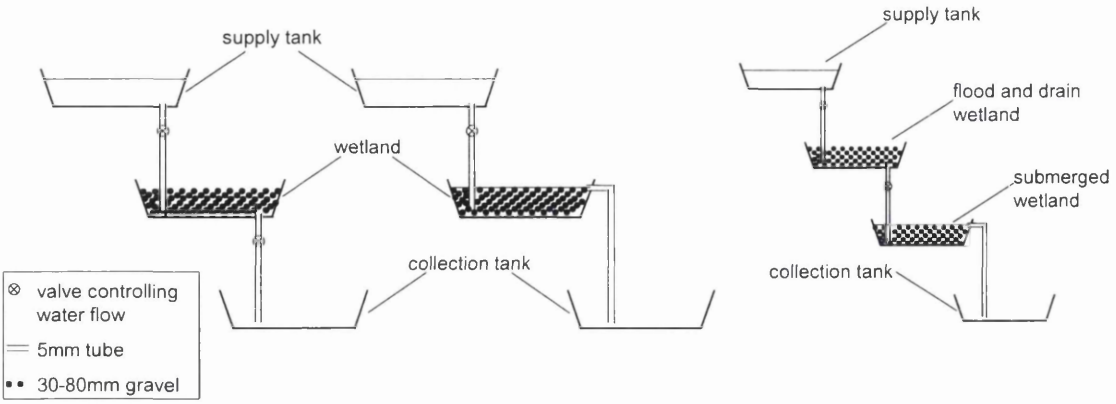
ranged from 600 to 2290 ppm during this period. This is equivalent to a maximum areal loading of 78.5g $N\text{-}NH_3/m^2$/day.

Samples of the microbial biofilm were collected at the same time as the outlet water samples. Water samples for analysis were taken immediately prior to the addition of new effluent (from now on described as "outlet"), and after thoroughly mixing the new effluent/ammonium chloride with the remaining water in the system ("inlet"). Samples were collected weekly and DNA for microbial analysis was extracted at the start of the experiment (week 0), and at the end of week 3, 6 (total bacterial community only), 7, 8 and 12.

2.5.2 Flood/drain wetland model design

Six model wetlands were used in this study. Each wetland consisted of one 8L plastic planting trough filled with limestone gravel (diameter 30-80mm) (Figure 2.3). The gravel was collected from the Selonda UK wetland. The wetlands were supplied with wastewater collected from the Centre for Sustainable Aquaculture Research, Swansea University. When necessary the salinity of the wastewater was adjusted to 30ppt before use. Experiments were carried out at ambient temperature (range -3°C - 23°C) between October 2006 and May 2007.

The wetlands were divided into two operational conditions: wetlands A-C were operated under submerged conditions, wetlands D-F were operated under flood/drain conditions. The experiment was divided into four different treatments. Treatments were run sequentially.



Flood and drain wetland: Drainage occurs by opening the valve on the wetland outflow

Submerged wetland: Overflow pipe ensures water can be recirculated with wetland remaining submerged

Combined wetland (treatment 4). Valves control flow through both wetlands

Figure 2.3 Diagram of the two modes of operation (flood/drain and submerged) used in the first three treatments in this study, and the combination wetland used in treatment four.

2.5.3 Description of experimental treatments in flood/drain experiment.

The following information about the different treatments used in this experiment is summarized in table 2.4.

Treatment 1 (Week 1-9). Wetlands received a daily application of wastewater (3L). Water passed once through the model and was then discarded. The three flood/drain wetlands had a daily drainage period of 10 minutes and were then re-flooding period with fresh effluent over a period of 4 hours. The water exchange in the three submerged wetlands also took 4 hours. On day one wastewater was added to all the wetlands. 24 hours later, and on subsequent days, water was drained from the flood/drain wetlands over a period of approximately ten minutes and discarded. Fresh effluent was added to the supply tank of all wetlands and allowed to empty into the wetland over a period of four hours. As the supply tanks emptied, the wastewater refilled the flood/drain cells or displaced the existing water

from the submerged cells. Water samples were taken for analysis from the inlet and outlet of the seventh cycle every week during treatment 1.

Treatment 2 (Weeks 10-15). Wetlands were operated as in treatment 1 except that water collected after passage through the wetland was not discarded, but used to refill the supply tank. On day one and day two, fresh effluent was supplied to the wetland, after day two water that had passed through the wetland was recirculated. The initial 2 x 3L wastewater inputs were recirculated for 7 days. Water samples were taken for analysis from the inlet on day 1 and outlet on day 7 (i.e. water that had made four passages through the wetland).

Table 2.4. Summary of water exchange conditions in the flood/drain and submerged wetlands in each of the four treatments of the study.

Treatment/ Mode of operation	Residence time	No. passages	Reflooding/ displacing time[1]	Treatment duration	Drainage time	Wetland identifiers
1 (single passage submerged)	1 day	1	4 hours	9 weeks	0 min	A - C
1 (single passage flood/drain)	1 day	1	4 hours	9 weeks	10 min	D - F
2 (long recirculated submerged)	7 days	4	4 hours	6 weeks	0 min	A - C
2 (long recirculated flood/drain)	7 days	4	4 hours	6 weeks	10 min	D - F
3 (short recirculated submerged)	7 days	4	4 hours	4 weeks	0 min	A - C
3 (short recirculated flood/drain)	7 days	4	10-15 min	4 weeks	10 min	D - F
4 (combined)	7 days	3	10-15 min	5 weeks	0 min	- - -

[1] Reflooding time is the length of time in flood/drain wetlands from the point at which the wetland is fully drained until the wetland is completely refilled. The displacing time is the time over which the water is replaced in the submerged wetlands

Treatment 3 (Weeks 16-19). As treatment 2, except that refilling time was reduced from 4 hours to 10-15 minutes i.e. total drain and refill cycle 20-25 min. Water samples were taken for analysis on from the inlet on day 1 and outlet on day 7 (four passages through the wetland).

Treatment 4 (weeks 20-24). In the final treatment the six individual wetlands were combined to create three two-wetland combination systems. These combination wetlands were created by connecting two wetlands in series (flood/drain followed by submerged). 6L effluent was added on day 1 in order to replace all treated wastewater from the wetlands. Water was recirculated from the outlet of submerged wetland to the inlet of flood/drain wetland. Refill time was kept at 10-15 minutes. Water samples were taken for analysis from the inlet (of the flood/drain wetland) on day 1 and outlet (of submerged wetland) on day 7 (three passages through the wetland).

During the experiment the areal loading was in the range of 0.13 to 5.37 $gN/m^2/day$.

2.5.4 Description of field site: Selonda UK constructed wetland

The field site for this study was a newly commissioned wetland on Anglesey, Wales. The wetland had been constructed to treat wastewater from a planned inland marine recirculation aquaculture facility with an annual production capacity of 1200 tonnes per annum. During the experimental period the wetland was supplied with effluent from a smaller existing marine fish farm (Bluewater Flatfish Farm Ltd), producing turbot (*Psetta maxima*),

with an annual production capacity of 130 tonnes per annum. Solid waste from the farm's ozonated "protein skimmers" and from backwashing of mechanical filters was released to a settlement tank. The supernatant from the settlement tank was then pumped on demand from the farm to the upper tier of the wetland and held in a storage tank. This water was periodically released under gravity from the holding tank to Geotube® containers (Bishop Aquatic Technologies, Ontario, Canada) under control of a float switch, thereafter passing into the wetland cells (see schematic representation in Figure 2.4). The Geotube® filters the water and collects solid contaminants. The wetland consisted of 14 individual cells, each approximately 180m^3 in volume, giving a total treatment area of around 2000m^2 (void space estimate 50.8%) The cells were organised in two rows of seven, with the second row being approximately 1.2m below the first. Each cell could be operated independently and manipulated to allow for either vertical or horizontal flow. The cells measured approximately 6m x 25m x 1.2m each, and were filled with three grades of limestone gravel (70-100mm at the bottom, with layers of 40-75mm and 25-40mm on top). Water was distributed across the wetland via a network of subsurface pipes, which released the effluent at the surface through vertical pipes.

To obtain time averaged water samples, siphons of 7mm tubing with valves to control water flow were inserted into the inlet of the upper cell and outlet of both cells and water was collected over the duration of each flood/drain cycle. Salinity during the period of this study was between 34 and 36ppt.

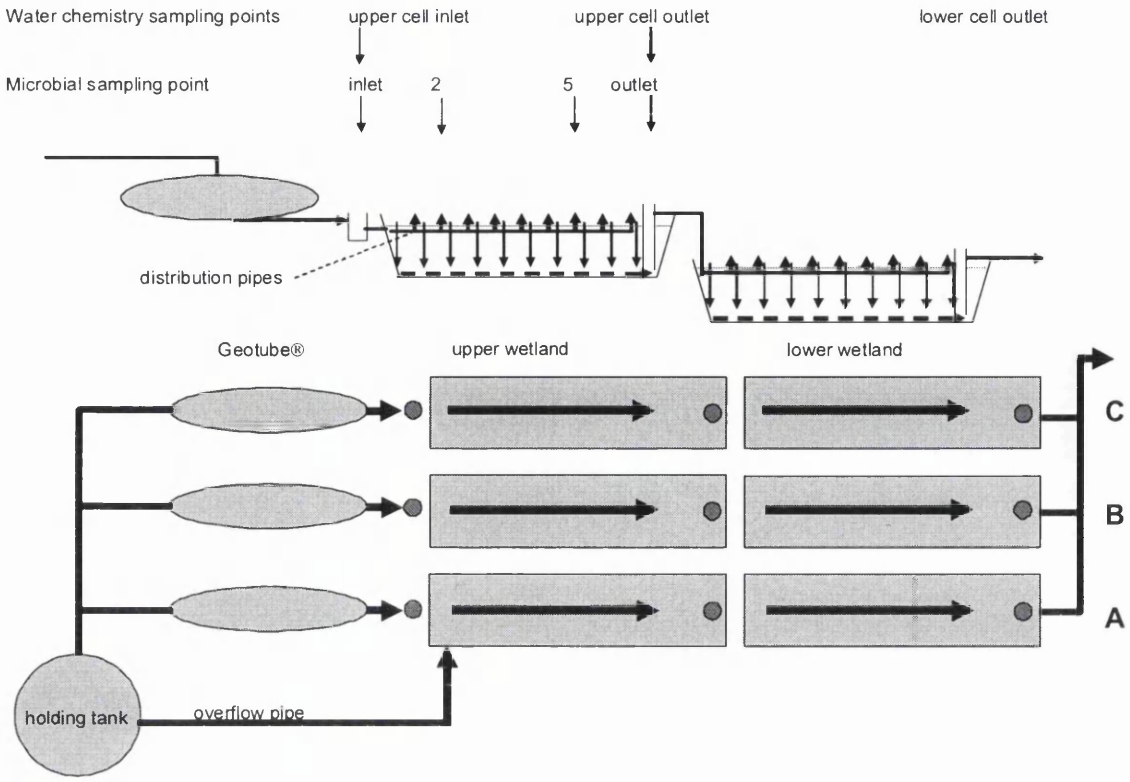


Figure 2.4. Schematic diagram of the fish farm wetland. Lower figure shows aerial view. At inlet and outlet collection points were samples taken from water flow. At collection points 2 and 5 samples were taken from the surface of the wetland media. Upper figure shows longitudinal section. Arrows indicate direction of water flow. The wetland consisted of a further 8 pairs of cells (located parallel to and beyond wetland C). At the time of the study the three pairs shown were in operation and were the only ones used for this study.

Chapter Three

Bacterial community responses to increasing ammonia concentrations in a model recirculating vertical trickle flow saline wetland

3.1 Introduction

3.1.1 Background information

Trickling filters are frequently used in the aquaculture industry to maintain low ammonia concentrations within recirculating systems (van Rijn, 1996) and are also commonly used in other wastewater treatments (Kadlec & Knight, 1996). The flow of water over the filter media draws in oxygen and promotes the growth of aerobic ammonia oxidizing bacteria and results in good rates of nitrification (Eding *et al.*, 2006). It is for this reason that a trickle flow model was used to test the maximum ammonia removal capacity of the wetlands.

When ammonia loading trials have previously been carried out on biological waste treatment systems, the eventual failure to remove ammonia appears to be linked to an imbalance with denitrification, and a build up of nitrite can occur associated with the failure (Burgess *et al.*, 2002; Shiskowski & Mavinic, 1998). It has been suggested that both a build up of nitrites or nitrates (poor denitrification) and pH changes accompanying increased denitrification can lead to nitrification failure (Shiskowski & Mavinic, 1998).

3.1.2 Experimental aims

This study was designed to provide information on likely treatment capacity and bacterial community structure in a newly constructed wetland at the site of a land-based marine fish farm. Given the novelty of the use of saline wetlands in aquaculture, it was considered important to test the ammonia

conversion capacity using a laboratory model. Since the wetland at the fish farm had been engineered to operate in a variety of management regimes, and given that low levels of oxygen limit nitrification in many wetlands, a recirculating vertical trickle flow regime was chosen for this study. Three replicate laboratory-scale wetlands were constructed and tested by dosing with aquaculture wastewater supplemented with increasing amounts of ammonium chloride. The aim of the study was to describe the changes to the total bacterial and ammonia oxidizing bacterial communities as the wetland was challenged with increasing ammonia concentrations and to establish the nitrification capacity of this system.

3.2 Results

3.2.1 Changes to ammonia, nitrite and nitrate concentrations during the experimental period

Three replicate model wetlands (A, B and C,) were designed for this study (see Chapter 2 Figure 2.2). Mean inlet ammonia concentration increased each week up to a maximum of 2050ppm in week 9. After week 9 the mean inlet concentration ranged from 753-1297ppm. Up to week 7 (maximum concentration of 358ppm $N\text{-}NH_3$), ammonia removal remained high (97.1-100% in each replicate) despite the weekly increases in ammonia concentration (Figure 3.1A). In week 8, when inlet ammonia concentration was 638-845ppm $N\text{-}NH_3$, ammonia removal fell in the wetlands to between 53.7-79.9%. Ammonia

removal was subsequently more variable (27.1-99.2%) than in the period up to week 7. Hereon, the period up to week 7 is described as period 1, and week 8 onwards is described as period 2. Mean ammonia removal in period 1 was significantly higher than in period 2 for each wetland (Mann Whitney U test $p<0.01$). (Nitrate and nitrite removal was not significantly different between periods. See Table A1 in appendix for p values.) Although the percentage of ammonia removed was lower in period 2, the total amount of ammonia removed was generally higher than in period 1, because of the higher starting concentration. In period 1, maximum ammonia removal was observed in week 7 when an initial ammonia concentration of 358ppm was reduced to 0.05ppm (99.99% removal). In period 2, a greater amount of ammonia was removed from all wetlands except for wetland C in week 12, when the concentration fell 325ppm from 1200ppm to 875ppm (44% removal). The maximum amount of ammonia removed (a reduction of 1280ppm from 2290ppm to 1010ppm) was observed in week 9 from wetland B, equivalent to 55.9% ammonia removal. The greatest amount of ammonia removed by wetlands A and C was also observed in week 9. Concurrent with the change in ammonia removal efficiency between week 7 and 8, accumulation of nitrite was observed in all wetlands (Figure 3.1B). Prior to this there was a trend for nitrite removal. During period 1 the maximum nitrite concentration was 5.7ppm (wetland C, week 6). In week 8 nitrite concentrations rose to 34.9-97.6ppm. Another large nitrite peak (25.5-82.6ppm) was recorded in week 10. Throughout period 2 outlet nitrite levels remained high compared to period 1.

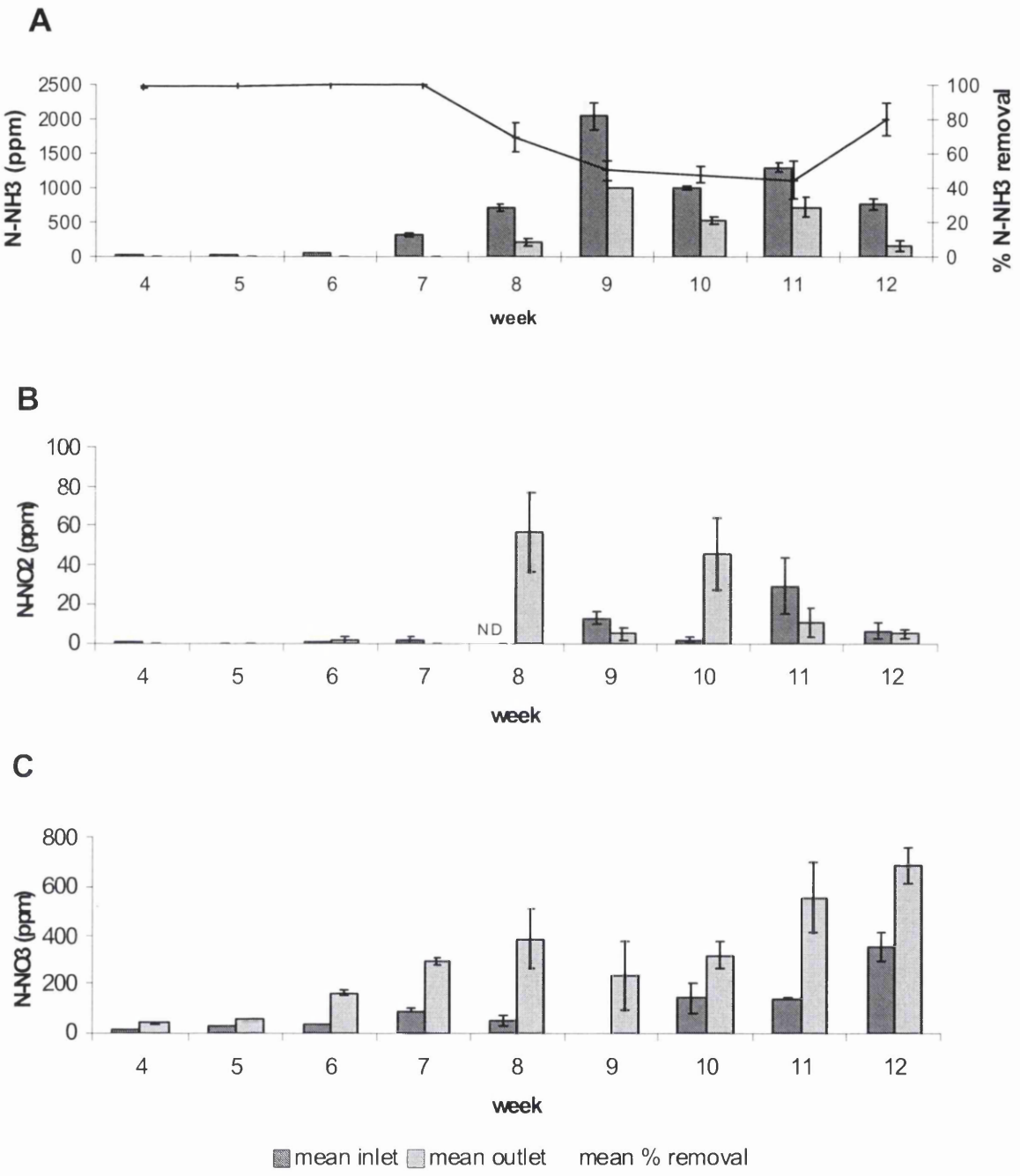


Figure 3.1. Inlet and outlet concentrations of ammonia (A), nitrite (B) and nitrate (C) for three replicate model wetlands. Mean inlet concentrations are indicated by dark grey bars and mean outlet concentrations are indicated by light grey bars. Line on ammonia graph shows percentage ammonia removed from water. Error bars =± 1SE (n=3) (ND= not determined)

Outlet nitrate concentration was generally higher than inlet concentration throughout the experiment, indicating poor denitrification (Figure 3.1C). From week 4 onwards nitrate concentrations increased progressively with time.

3.2.2 Increasing ammonia concentration results in a decrease in diversity of the bacterial community

The total number of detectable bacterial OTUs increased during the first seven weeks as measured by T-RFLP of 16s rDNA sequences (Figures 3.2 and 3.3). At the start of the experiment the mean number of detectable bacterial OTUs was 19.3 (Figure 3.3) increasing to a maximum of 32.7 in week 7. The number of OTUs common to all three replicates (consensus) was 6 at the start of the experiment and increased to 18 in week 7. The number of bacterial OTUs subsequently decreased during period 2 (mean = 10.7, consensus OTUs = 1, week 8). Between weeks 8 and 12 the mean number of OTUs observed fell to 8.7. After week 8 the number of OTUs in wetlands A and C fell from 14 and 13 respectively to 6 in both. In week 12, near complete ammonia removal (99.2%) was achieved in one replicate (B); this was coincident with a greater number of bacteria (14 OTUs) being detected in this wetland than the other two replicates. Analysis of T-RFLP fragments common to all replicates (Figure 3.4) showed a trend that once bacterial OTUs established themselves in the biofilm they persisted until the inlet ammonia concentration increased above 358ppm.

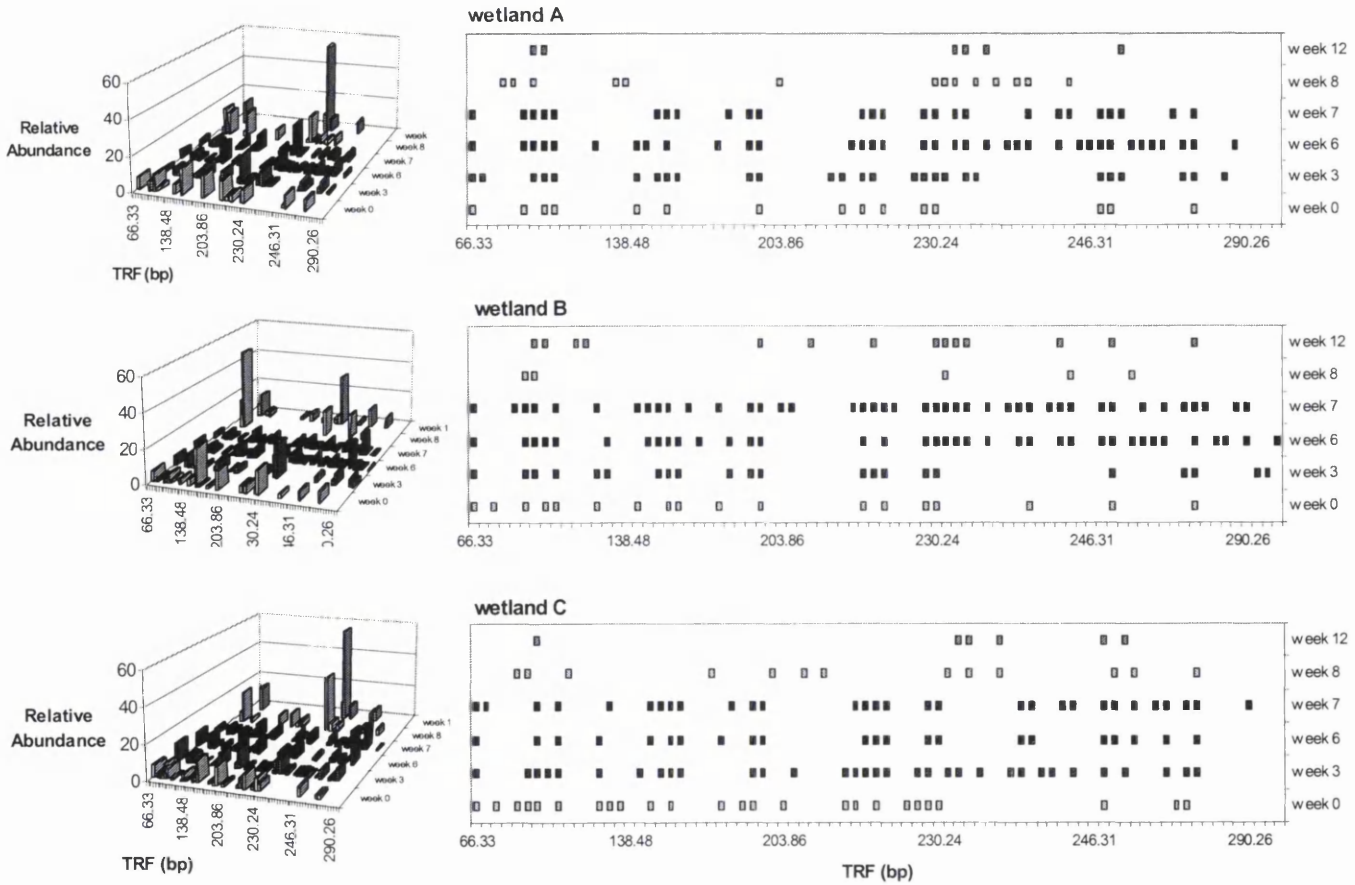


Figure 3.2. Bacterial community structures for each wetland throughout the experiment. Graphs on left show the relative abundance of TRFs detected by T-RFLP of *16s rRNA* gene, graphs on the right show the same data displayed as presence/absence data

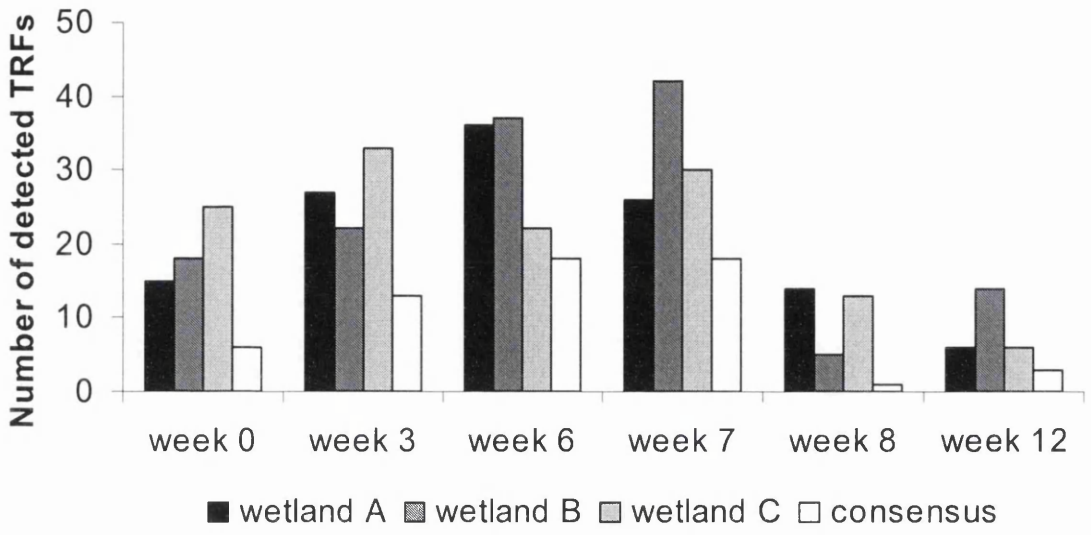


Figure 3.3. The number of bacterial OTUs detected in the wetlands in different weeks. The number of OTUs was measured by the number of TRFs obtained with 16s rDNA T-RFLP. Wetland A (black), wetland B (dark grey), wetland C (light grey) consensus (OTUs common to all three wetlands) (white).

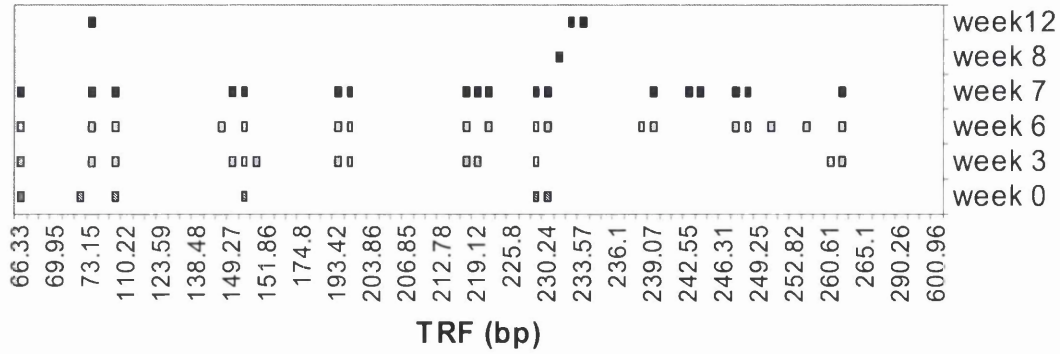


Figure 3.4. The changing patterns of bacterial OTUs found in the wetlands over the course of the experiment. Only those TRFs obtained by 16s rDNA T-RFLP that were found in all three replicate wetlands are shown.

3.2.3 A succession of ammonia oxidizing bacteria dominate the wetlands

Terminal restriction fragments (TRFs) of the *amoA* gene were monitored in samples. Three major TRFs, representing at least three different species of ammonia oxidizing bacteria, dominated the communities in all replicate systems at different time points (Figure 3.5). Other TRFs were observed but these were neither the most dominant OTUs nor were they detected in samples from all three wetlands at any particular time point. None of the minor TRFs corresponded to predicted TRF sizes based on the *in silico* restriction digests of known *amoA* gene sequences (Figure 3.6). Of the three dominant OTUs, the first, represented by a fragment of 175bp, had its maximum relative abundance in week 0. As the experiment progressed, this OTU decreased in relative abundance and a second OTU represented by a fragment of 485bp became more dominant. This reached its maximum relative abundance in week 7 in all wetlands. From week 8 onwards the third OTU, represented by a TRF of 337bp, dominated. This OTU had a maximum relative abundance in week 12 in all replicates.

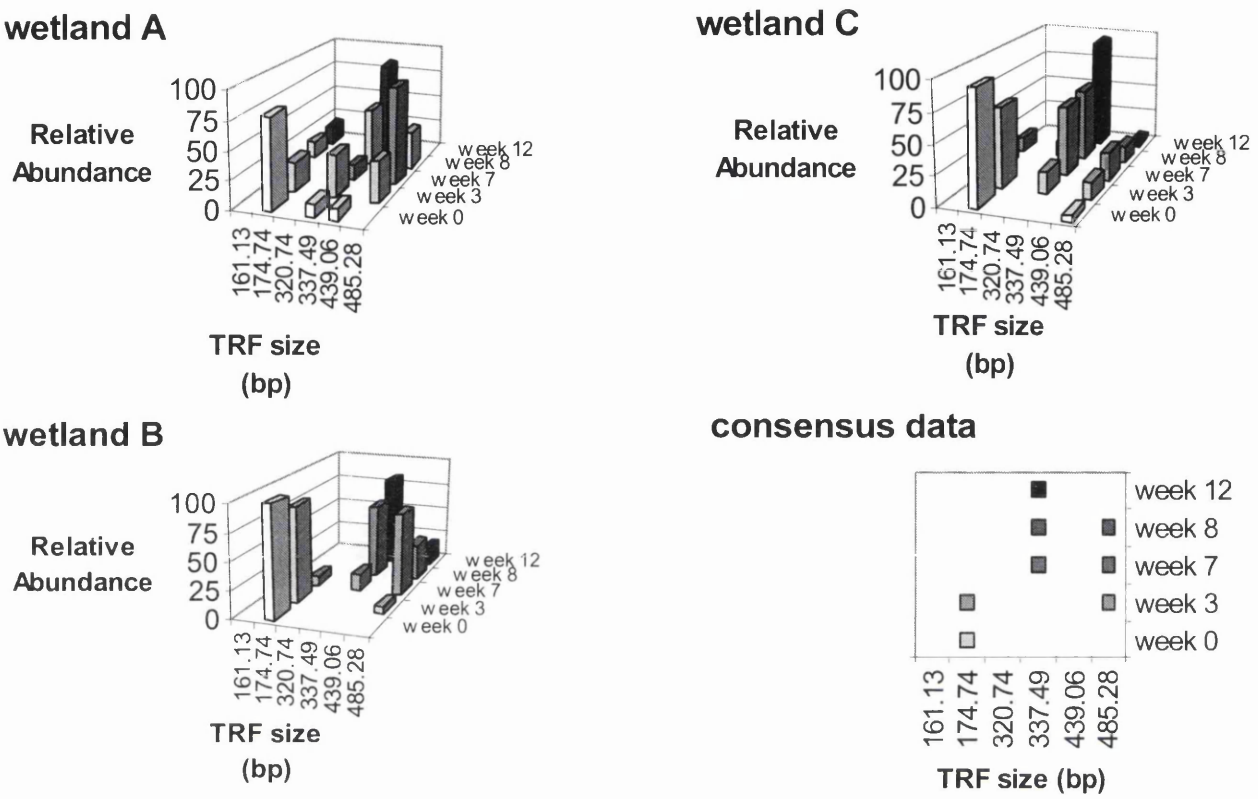
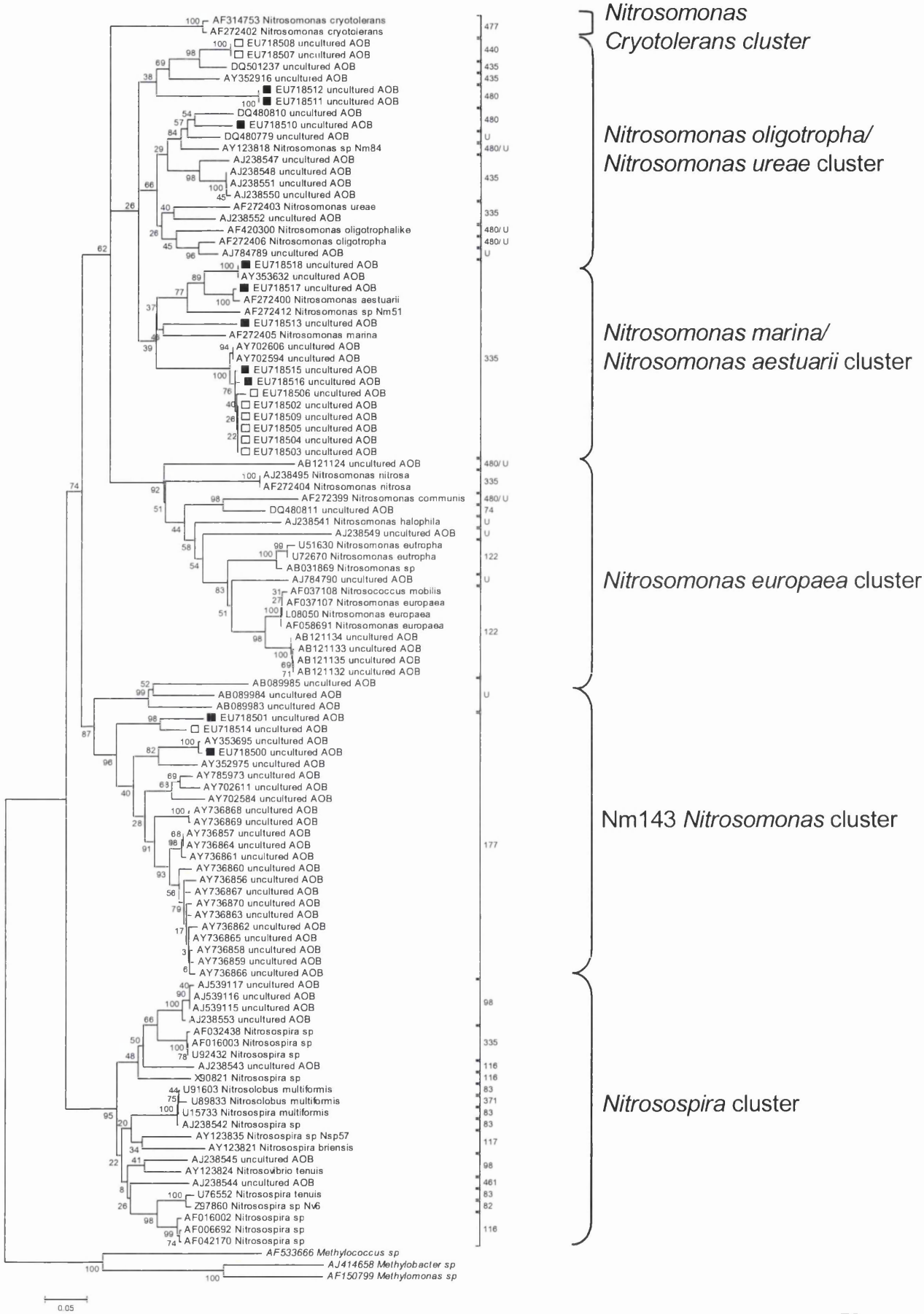


Figure 3.5. *amoA* TRFs detected in the wetlands in different weeks. Top left: relative abundance of TRFs in wetland A. Lower left Relative abundance in wetland B. Top right: relative abundance in wetland C. Lower right: Consensus data (fragments occurring in all three replicates.)

Figure 3.6. (Page 72) Neighbour joining tree of *amoA* sequences with predicted TRF sizes indicated. (Species names maintained as stated on the GenBank entry even when official names have changed.) Black squares indicate samples taken from model wetlands. White squares indicate samples taken from the Selonda UK wetland (Chapter 5). Three particulate methane monooxygenase gene (pMMO) sequences from methane oxidizing bacteria were used as an out group.


AF314753 Nitrosomonas cryotolerans
AF272402 Nitrosomonas cryotolerans
EU718508 uncultured AOB
EU718507 uncultured AOB
DQ501237 uncultured AOB
AY352916 uncultured AOB
EU718512 uncultured AOB
EU718511 uncultured AOB
DQ480810 uncultured AOB
EU718510 uncultured AOB
DQ480779 uncultured AOB
AY123818 Nitrosomonas sp Nm84
AJ238547 uncultured AOB
AJ238548 uncultured AOB
AJ238551 uncultured AOB
AJ238550 uncultured AOB
AF272403 Nitrosomonas ureae
AJ238552 uncultured AOB
AF420300 Nitrosomonas oligotrophalike
AF272406 Nitrosomonas oligotropha
AJ784789 uncultured AOB
EU718518 uncultured AOB
AY353632 uncultured AOB
EU718517 uncultured AOB
AF272400 Nitrosomonas aestuarii
AF272412 Nitrosomonas sp Nm51
EU718513 uncultured AOB
AF272405 Nitrosomonas marina
AY702606 uncultured AOB
AY702594 uncultured AOB
EU718515 uncultured AOB
EU718516 uncultured AOB
EU718506 uncultured AOB
EU718502 uncultured AOB
EU718509 uncultured AOB
EU718505 uncultured AOB
EU718504 uncultured AOB
EU718503 uncultured AOB
AB121124 uncultured AOB
AJ238495 Nitrosomonas nitrosa
AF272404 Nitrosomonas nitrosa
AF272399 Nitrosomonas communis
DQ480811 uncultured AOB
AJ238541 Nitrosomonas halophila
AJ238549 uncultured AOB
U51630 Nitrosomonas eutropha
U72670 Nitrosomonas eutropha
AB031869 Nitrosomonas sp
AJ784790 uncultured AOB
AF037108 Nitrosococcus mobilis
AF037107 Nitrosomonas europaea
L08050 Nitrosomonas europaea
AF058691 Nitrosomonas europaea
AB121134 uncultured AOB
AB121133 uncultured AOB
AB121135 uncultured AOB
AB121132 uncultured AOB
AB089985 uncultured AOB
AB089984 uncultured AOB
AB089983 uncultured AOB
EU718501 uncultured AOB
EU718514 uncultured AOB
AY353695 uncultured AOB
EU718500 uncultured AOB
AY352975 uncultured AOB
AY785973 uncultured AOB
AY702611 uncultured AOB
AY702584 uncultured AOB
AY736868 uncultured AOB
AY736869 uncultured AOB
AY736857 uncultured AOB
AY736864 uncultured AOB
AY736861 uncultured AOB
AY736860 uncultured AOB
AY736856 uncultured AOB
AY736867 uncultured AOB
AY736870 uncultured AOB
AY736863 uncultured AOB
AY736862 uncultured AOB
AY736865 uncultured AOB
AY736858 uncultured AOB
AY736859 uncultured AOB
AY736866 uncultured AOB
AJ539117 uncultured AOB
AJ539116 uncultured AOB
AJ539115 uncultured AOB
AJ238553 uncultured AOB
AF032438 Nitrosospira sp
AF016003 Nitrosospira sp
U92432 Nitrosospira sp
AJ238543 uncultured AOB
X90821 Nitrosospira sp
U91603 Nitrosolobus multiformis
U89833 Nitrosolobus multiformis
U15733 Nitrosospira multiformis
AJ238542 Nitrosospira sp
AY123835 Nitrosospira sp Nsp57
AY123821 Nitrosospira briensis
AJ238545 uncultured AOB
AY123824 Nitrosovibrio tenuis
AJ238544 uncultured AOB
U76552 Nitrosospira tenuis
Z97860 Nitrosospira sp Nv6
AF016002 Nitrosospira sp
AF006692 Nitrosospira sp
AF042170 Nitrosospira sp
AF533666 Methylococcus sp
AJ414658 Methylobacter sp
AF150799 Methylomonas sp
0.05
Nitrosomonas Cryotolerans cluster
Nitrosomonas oligotropha/ Nitrosomonas ureae cluster
Nitrosomonas marina/ Nitrosomonas aestuarii cluster
Nitrosomonas europaea cluster
Nm143 Nitrosomonas cluster
Nitrosospira cluster

3.2.4 The dominant ammonia-oxidising bacteria are nitrosomonad species

In silico restriction digests of 84 *amoA* sequences from the GenBank database (Figure 3.6) allowed a tentative identification of the three dominant OTUs. Observed T-RFLP fragments are known to differ slightly from predicted sizes due to base composition (Kaplan & Kitts, 2003). To confirm that the TRFs seen in the sample profiles correspond to the taxons identified by *in silico* digests, cloned fragments of *amoA* of known sequence were submitted to fragment analysis, and the fragment size compared to sample profiles. A cloned *amoA* fragment of known sequence with a predicted TRF of 177bp produced an observed TRF of 174.74. A clone with predicted size of 335bp produced a TRF of 337bp. A predicted TRF of 480bp corresponds to an observed TRF of 485bp (Figure 3.7). On the basis of TRF size, the OTU dominant at low ammonia concentrations (TRF=174.74) was assigned to the so called "novel AOB lineage of *Nitrosomonas*" or Nm143 lineage (Purkhold *et al.*, 2003). Sequencing of 2 cloned PCR products with an observed TRF of 175bp derived from wetlands samples, revealed close homology with *amoA* sequences of the Nm143 lineage. The OTU most abundant in week 7 (TRF=485.28) is most likely to have come from the *N.oligotropha/N.ureae* cluster. Sequences of 3 cloned PCR products with an observed TRF of 485.28pb derived from wetland samples revealed homology with sequences from the *N.oligotropha/N.ureae* cluster (Figure 3.6 shows that two of the sequences (EU718512 and EU718511) are equally similar to the *N.aestuarii/N.marina* cluster as the *N.oligotropha/N.ureae* cluster.

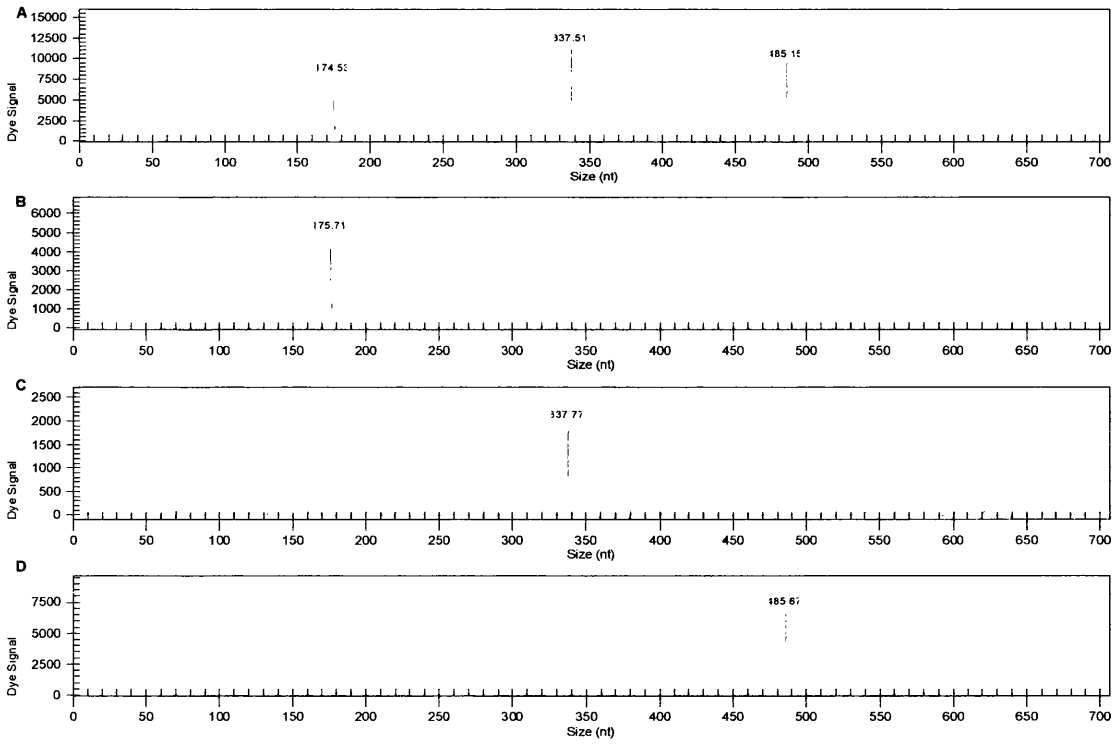


Figure 3.7 Fragment analysis profiles comparing a wetland DNA sample with cloned a*moA fragments of known sequence.* A) T-RFLP profile from wetland A week 3. B) EU718500 taken from model wetland during start up period. Predicted TRF based on sequence = 177bp. C) EU718504 obtained from full scale wetland. Predicted TRF= 335bp D) EU718511 taken from model wetland during start up period. Predicted TRF=480bp.

However as a predicted 480bp TRF occurs within the *N.oligotropha/N.ureae* cluster, but all the examples in the *N.aestuarii/N.marina* cluster have a TRF of 335bp, these two sequences were classified as *N.oligotropha/N.ureae*.) The OTU dominant in later weeks with a TRF of 337bp was assigned to the *N.aestuarii/N.marina* cluster. *Nitrosospira* sp and *N.ureae* also produce the same sized terminal fragments, but sequencing of 5 cloned PCR products with TRF of 337bp derived from wetlands samples, revealed

close homology with *amoA* sequences of *N.aestuarii/N.marina.* This method cannot give a definitive identification but some confidence in this assessment comes from the observations that all *amoA* sequences obtained from the model had terminal restriction fragment sizes that were observed in T-RFLP profiles.

3.3 Summary

The model vertical trickle flow wetlands were supplied with aquaculture wastewater supplemented with increasing amounts ammonia (up to 2290ppm) in the inlet water. On the basis of the removal of ammonia the experiment was divided into two periods. During period 1, ammonia concentration increased to a maximum of 358ppm, and in excess of 97.1% ammonia removal was observed. The mean number of bacterial OTUs detected by T-RFLP increased during this period from 19.3 to 32.7. In period 2, when mean inlet ammonia concentration was between 638ppm and 2050ppm, the mean percentage of ammonia removed was lower, but the maximum amount of ammonia removed (a reduction in concentration of 1280ppm) was observed in week 9. An increase in nitrite outlet concentration and a decrease in the number of bacterial OTUs were noted at the beginning of period 2 (week 8). The number of bacterial OTUs further declined from 10.7 to 8.7 between weeks 8 and 12. A succession of dominant ammonia oxidizing OTUs was seen in the wetlands. A TRF corresponding to Nm143 lineage was present in all wetlands up to week 3, from

week 3 to week 8 a TRF corresponding to *Nitrosomonas oligotropha/N.ureae* was present in all wetlands, and from week 7 to 12 *N.Marina/N.aestuarii* was present in all wetlands

Although this model demonstrated the capacity of constructed wetlands to treat saline wastewater with considerably higher concentrations of ammonia than have been previously measured in aquaculture effluent, the constant recirculation of water through the model and range of water temperatures (24.5°C to 28°C), are likely to have increased nutrient removal above that which could be expected in the normal operation of a constructed wetland in a temperate climate. In order to mimic more realistic operating conditions, the second model (details in Chapter 4) was located outdoors and had much lower water recirculation.

T-RFLP is able to identify changes in the composition of total bacterial and ammonia oxidizing bacterial communities, but cannot describe the changes in number or activity of bacteria. If the T-RFLP data had been paired with quantitative- or real time- PCR it would have been possible to answer further questions about the bacterial communities in the wetlands, such as:

- Was the decrease in the number of bacterial OTUs between week 7 and 8 accompanied by a decrease in the total number of bacterial cells?
- How did the number of ammonia oxidizing bacteria change as the ammonia concentration increased and the dominant AOB OTU changed?

- Did the ammonia oxidizing activity (as measured by gene expression of *amoA*) increase even when the percentage of ammonia removed decreased after week 7?

Chapter Four

Balancing microbial nitrification and denitrification in a model saline wetland system treating aquaculture wastewater

4.1 Introduction

4.1.1 Previous work indicates flood/drain cycles increase nitrogen removal from constructed wetlands

Several laboratory studies have shown that operating freshwater wetlands by alternately flooding the cells with wastewater and then draining them enhances the nitrogen removal compared to conventional surface flow wetlands (e.g. Austin *et al.*, 2003; Tanner *et al.*, 1999; Zhao *et al.*, 2004). To date no studies of flood/drain cycles in constructed wetlands treating saline wastewater have been published.

Increased nitrogen removal in flood/drain wetlands has been explained in terms of adsorption and desorption of ions to the wetland media (Austin *et al.*, 2003; Tanner *et al.*, 1999). It is widely accepted that metabolically active bacteria have a strong propensity to adhere to surfaces, and that the protected environment of the biofilm gives attached bacteria advantages over planktonic bacteria (Costerton *et al.*, 1995; Stoodley *et al.*, 2002). Therefore the region in contact with the wetland media is critical for nutrient conversions. During the flood period there is maximum contact between the media and dissolved ions in the bulk water, and cation exchange sites on the surface of the media and organic matter in the wetland become occupied by ions (e.g. NH_4^+) present in the wastewater. During the drainage period, oxygen rapidly penetrates the biofilm providing suitable conditions for the nitrification of ammonia to nitrate by aerobic nitrifiers (Kadlec & Knight, 1996). For efficient ammonia removal it is important that the drained period is long enough to allow oxygen transfer to the biofilm

(Zhao *et al.*, 2004). When the wetland is flooded, available oxygen is limited and denitrifying bacteria are able to reduce nitrates and nitrites to gaseous nitrogen products. With cation exchange sites now vacant, more ammonium ions from the bulk water can adsorb to the media. Clearly, in this scenario the amount of ammonia that can be removed is limited by the adsorption capacity of the media and repeated cycles will be beneficial, particularly where adsorption capacity is low. Previous freshwater wetland studies have reported that removal of organic nitrogen and ammonium increased with increasing frequency of flood/drain cycles (up to 16 d^{-1}), but also observed nitrate accumulation with a high cycle number, suggesting disruption to the denitrification processes (Tanner *et al.*, 1999). Similarly Austin (2003) found denitrification to be sensitive to cycle number.

In a laboratory scale study of a freshwater flood/drain wetland, *Nitrosospira tenuis, Nitrosomonas marina, N.oligotropha, N.communis, N.eutropha/N.europa, N.mobilis and N.cryotolerans* were all identified, with the proportion of autotrophic ammonia oxidizers and nitrite oxidizers decreasing by 68% and 63% respectively over the first 36 months of operation (Austin *et al.*, 2003). Other organisms such as *Paracoccus denitrificans* (an aerobic denitrifier/ heterotrophic nitrifier) may therefore have an increased role in nitrification and denitrification in flood/drain wetlands, and oxygen tolerant facultative denitrifiers such as *Zooglea ramigera, Pseudomonas* sp. and *Alcaligenes* sp. have been identified as important species in model flood/drain wetlands (Austin *et al.*, 2003; Maciolek & Austin, 2006).

4.1.2 Experimental aims

The aim of this experiment was to identify whether the previously reported increases in nitrification and denitrification in freshwater wetlands could be replicated in saline model wetlands, and to investigate the differences in bacterial communities that develop in flood/drain and submerged wetlands. The performance of three replicate submerged wetlands was compared to three flood/drain wetlands under three different treatment regimes. Performance of a combined flood/drain and submerged wetland was also investigated.

4.2 Results

4.2.1 Multiple flood/drain cycles improve total Kjeldahl nitrogen (including ammonia and organic nitrogen) removal

Full descriptions of the different treatments can be found in Chapter 2 (section 2.5.3 and Table 2.3). During treatment 1 (single passage) both the submerged and flood/drain wetlands showed relatively low removal of TKN (including both organic nitrogen and ammonia) compared to later treatments, with up to 46% TKN removal, 25% ammonia removal and 68% organic nitrogen removal being observed in the flood/drain wetlands in treatment 1. Removals were very similar in both conditions, with no significant differences between flood/drain and submerged wetlands being observed (Figure 4.1). In treatments 2 and 3 (long and short recirculation treatments), when the residence time increased, an improvement in TKN, ammonia and organic

nitrogen removal was observed in the flood/drain wetlands. Up to 87.0% TKN removal, 99.8% ammonia removal and 91.3% organic nitrogen removal were seen during treatment 2 and up to 87.6% TKN, 99.9% ammonia and 97.2% organic nitrogen were seen in treatment 3. The outlet concentration of TKN and ammonia was significantly lower during treatment 2 in the flood/drain wetlands than in the submerged wetlands (both Mann-Whitney U $p<0.05$). In treatment 3 the TKN, ammonia and organic nitrogen concentrations were lower in the flood/drain wetlands (all Mann-Whitney U $p<0.05$). (All p values show in appendix Table A2). Removal was high in treatment 4, in particular with consistently high removal of ammonia being achieved (84.7-99.9%). TKN and Organic nitrogen removal remained high at up to 92% and up to 93% respectively. The outlet concentrations of TKN, ammonia and organic nitrogen were significant lower than the inlet concentration for all wetlands except for ammonia in the submerged wetland under treatment 1 (see Figure 4.1 and appendix Table A3 for significance levels).

4.2.2 Nitrate and nitrite removal processes are adversely affected by multiple flood/drain cycles

Nitrite removal was observed in both conditions during the first treatment (Wilcoxon $p<0.01$), but there was no significant difference between the conditions (Figure 4.1). In all subsequent treatments, nitrite concentration increased for both modes of operation, the increase being greater in the flood/drain wetlands, although no significant difference was detected using a Mann-Whitney U test. High median nitrite concentrations

(0.56 – 3.02ppm) were measured in all outlet water in treatments 2 and 3, and a maximum nitrite concentration of 7.5ppm was recorded in one of the flood/drain wetlands in treatment 3. Due to input nitrite concentrations being very low or undetectable, the very high percentage increases that resulted were not helpful for comparison of treatments. However treatment 4 was notable for the low nitrite outlet concentrations and having the smallest median increase in nitrite of all the recirculated treatments. Median outlet nitrite concentration in treatment 4 was 0.09ppm.

Nitrate was removed in both conditions during treatments 1 and 3 (Wilcoxon $p \leq 0.01$), but in treatment 2 and 4 non-significant increases were observed in both conditions. Outlet nitrate concentration was significantly lower in the submerged wetlands in treatments 2 and 3 (Mann-Whitney U $p<0.01$).

4.2.3 Improvements in TKN removal are balanced by reduced nitrate/nitrite removal

Overall, it was apparent that total nitrogen (TN) concentration of the outlet water was not significantly different between the two operational conditions during any treatment (Figure 4.1), possibly because the conditions that increased TKN removal decreased nitrate/nitrite removal. Total nitrogen removal was significant (Wilcoxon $p<0.01$) in all treatments except treatment 2 (see Figure 4.1 and Table A3 in appendix for significance levels). Pearson's correlation coefficient (r) was calculated to measure the effect size of the experimental condition (flood/drain or submerged). The effect size for each of the measured forms of nitrogen (when a significant difference in

outlet concentration was observed) was largest in treatment 3 and smallest in treatment 1 (r values can be found in appendix table A2). In the best performing treatment (treatment 3), 64.2% of the variance in nitrate concentration (i.e. r^2=0.642), 26.8% of the variance in organic nitrogen concentration, 18.0% of the variance in ammonia concentration and 23.3% of the variance in TKN concentration could be explained by the experimental condition. (No significant difference in outlet concentration of total nitrogen or nitrite was observed in treatment 3. For all cases where the difference between flood/drain and submerged outlet concentration was significant, at least a medium effect (Cohen, 1992) was observed (r<-0.3). For organic nitrogen in treatment 3 and nitrate in treatments 2 and 3 a large effect was observed (r<-0.5).

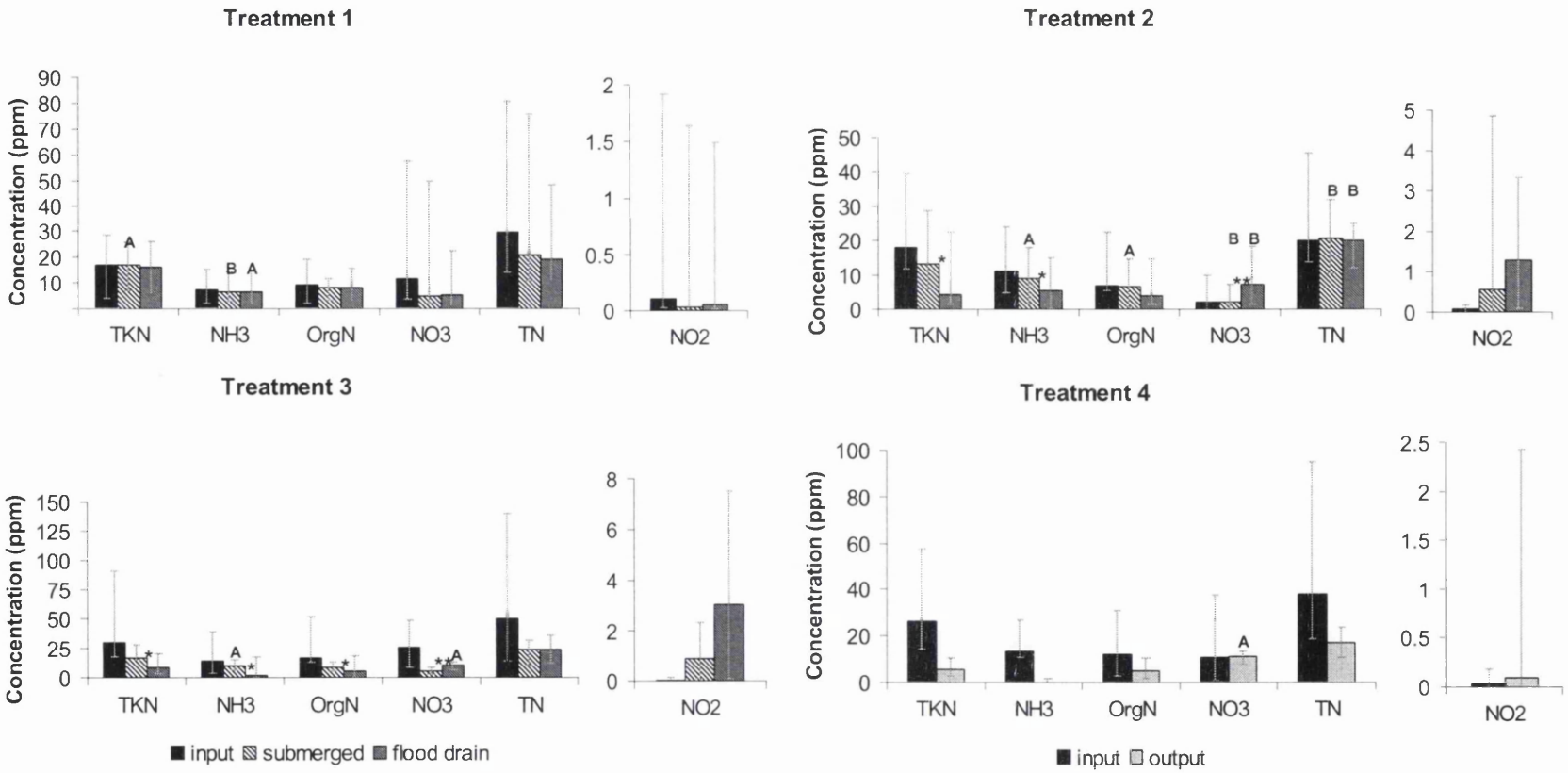


Figure 4.1. Median inlet and outlet concentrations of different nitrogen species in model flood/drain and submerged wetlands for the four different treatments. Error bars show range. Significant differences between the outlet concentration of the submerged and flood/drain wetlands are marked * for values $p<0.05$, and ** for $p<0.01$ (Mann-Whitney U test). Change between inlet and outlet concentration was significant ($p<0.01$) except where marked A ($p<0.05$) or B (not significant, Wilcoxon test). TKN =total Kjeldahl nitrogen, NH3 = ammonia, OrgN = organic nitrogen, NO3 = nitrate, TN = total nitrogen, NO2 = nitrite

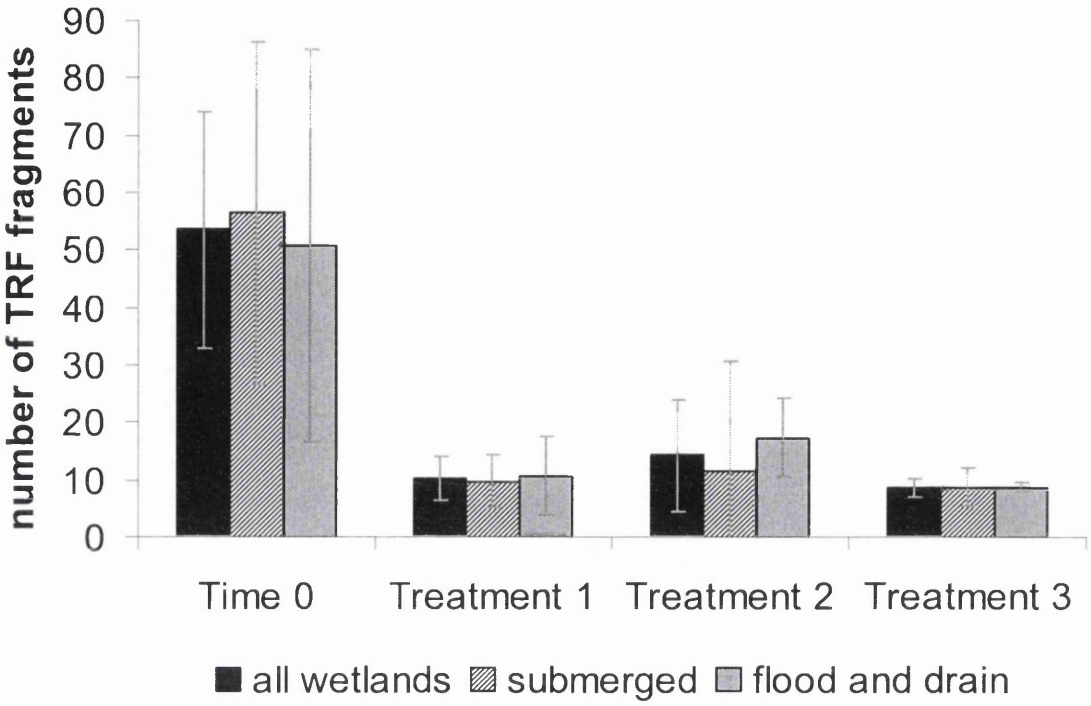


Figure 4.2. Mean number of bacterial OTUs found in the wetlands for each treatment. The mean number of OTUs was determined by number of terminal restriction fragments obtained with 16s rRNA T-RFLP. Error bars = ±2SE (n=3)

4.2.4 Total bacterial communities show a greater degree of similarity in submerged wetlands

The number of bacterial OTUs present in submerged, flood/drain and all wetlands at time 0 was significantly higher than the number of OTUs found in later weeks (Figure 4.2, Mann-Whitney U test $p<0.01$). No other statistically significant differences between treatments were observed, nor were there any significant differences between the two modes of wetland operation.

The change in bacterial community structure number after time 0 is further illustrated by principle component analysis (PCA). Separation by the first two principal components revealed that at time 0, the bacterial communities were diverse but could be distinguished from those sampled in later weeks (Figure 4.3). After time 0, the bacterial communities from the submerged wetlands clustered together, with the exception of one sample from treatment 1 (code 1B). The flood/drain community was more diverse (showed greater dissimilarity on the PCA plot); samples from wetland D and E in treatment 3 lay within the submerged cluster but the remaining samples were spread throughout the plot.

T-RFLP profiles revealed that most of the dominant bacteria OTUs were not specific to a particular treatment or mode of operation (Figure.4 4). The dominant OTU in most samples was represented by a 72bp TRF which occurred throughout the experiment. This OTU formed a major part of the community in both the submerged and flood/drain wetlands. Two OTUs represented by fragments of 239bp and 249bp tended to be more dominant, though not exclusive to the flood/drain wetlands.

4.2.5 The dominant ammonia oxidizing OTUs are different in flood/drain and submerged wetlands

PCA of ammonia oxidizing bacterial communities revealed appreciable diversity at time 0 (Figure 4.5), with a distinct community emerging in the flood/drain wetlands during the study, from treatment 2 onwards. The flood/drain and submerged communities became distinct from one another. The submerged communities did not form as tight a cluster as the flood/drain

communities. In the flood/drain mode of operation, the initial communities developed towards a profile in which the dominant bacterial OTU was represented by a 337bp TRF and the only other detectable OTU was represented by a 497bp TRF (Figure 4.6). The 337bp OTU was present in one flood/drain wetland (D) throughout the study, and appeared in the remaining flood/drain wetlands (E and F) during treatments 2 and 3. This OTU appeared to become dominant at the expense of an OTU with a TRF of 107bp. In most wetlands at the start of the experiment, this 107bp TRF was dominant (a 485bp TRF was dominant in wetland B, and a 337bp TRF was dominant in wetland D). This OTU eventually disappeared from the flood/drain wetlands, but persisted in the submerged wetlands, being present in all submerged wetlands in treatment 3. The 497bp TRF observed during treatment 3 represents the full length *amoA* PCR product. This fragment persisted even when samples were re-digested with excess enzyme (*Hph*I). It was a component of all the flood/drain wetlands, but only present in one of the submerged wetlands. A 100bp fragment appeared in four out of the six wetlands at the beginning of the experiment, at relatively low abundance (up to 12.25%), but did not appear in later profiles except in wetland C in treatment 3, and wetlands A and E in treatment 1 (in the latter wetland it made up 31% of the ammonia oxidizing population).

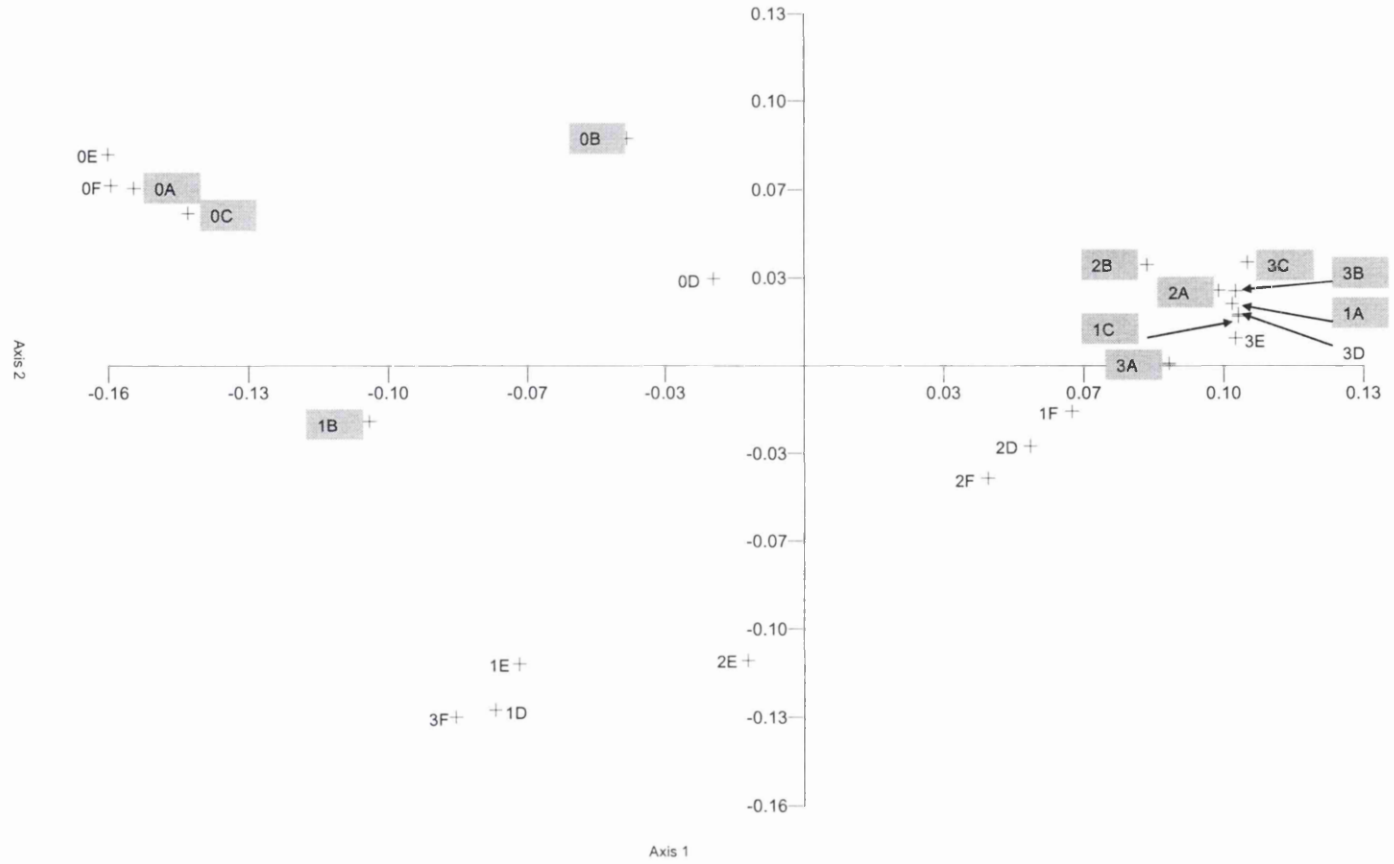


Figure 4.3. Principal component analysis of chord transformed 16s rRNA T-RFLP data, showing the degree of similarity between different bacterial communities. Key to data labels: Number indicates treatment (0= time 0), letter indicates wetland identifier –A-C (highlighted) are submerged wetlands, D-F are flood/drain wetlands. First two axes account for 55% variance.

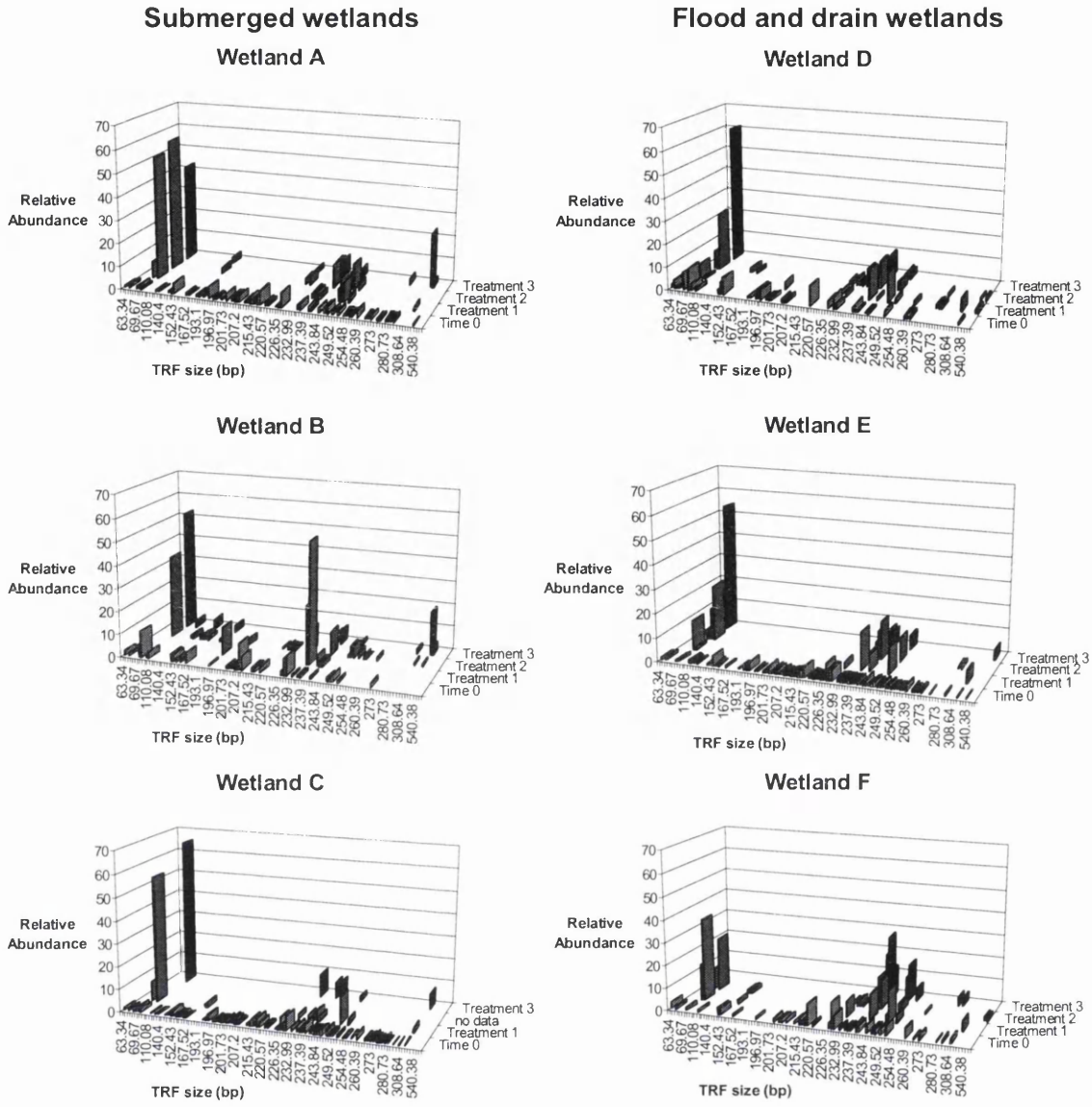


Figure 4.4. Bacterial community structures for each wetland in different treatments. Graphs show the relative abundance of TRFs detected by T-RFLP of *16s rRNA* gene

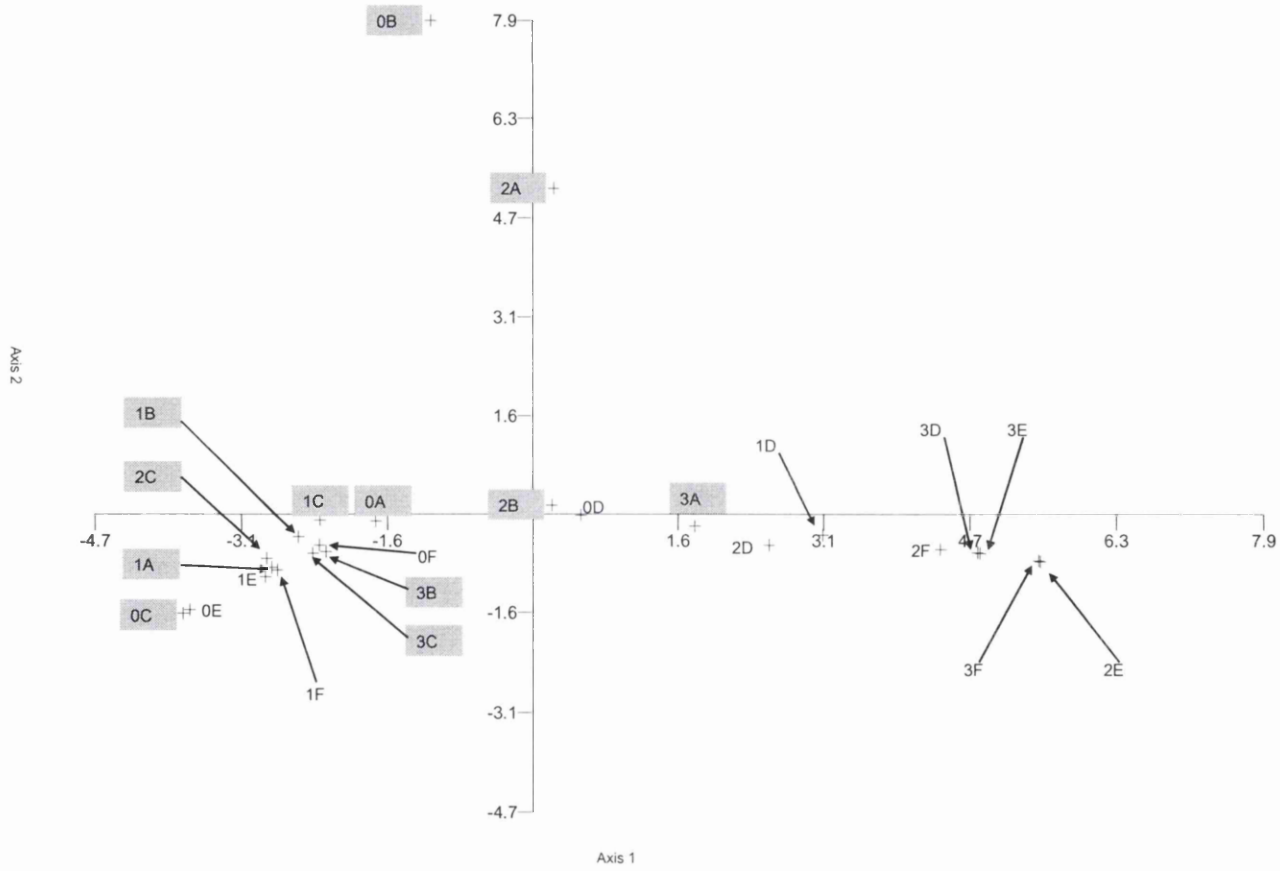


Figure 4.5. Principal component analysis of chord transformed *amoA* T-RFLP data, showing the degree of similarity between different ammonia oxidizing bacterial communities. Key to data labels: Number indicates treatment (0=time 0), letter indicates wetland identifier –A-C (highlighted) are submerged wetlands, D-F are flood/drain wetlands. First two axes account for 87% variance.

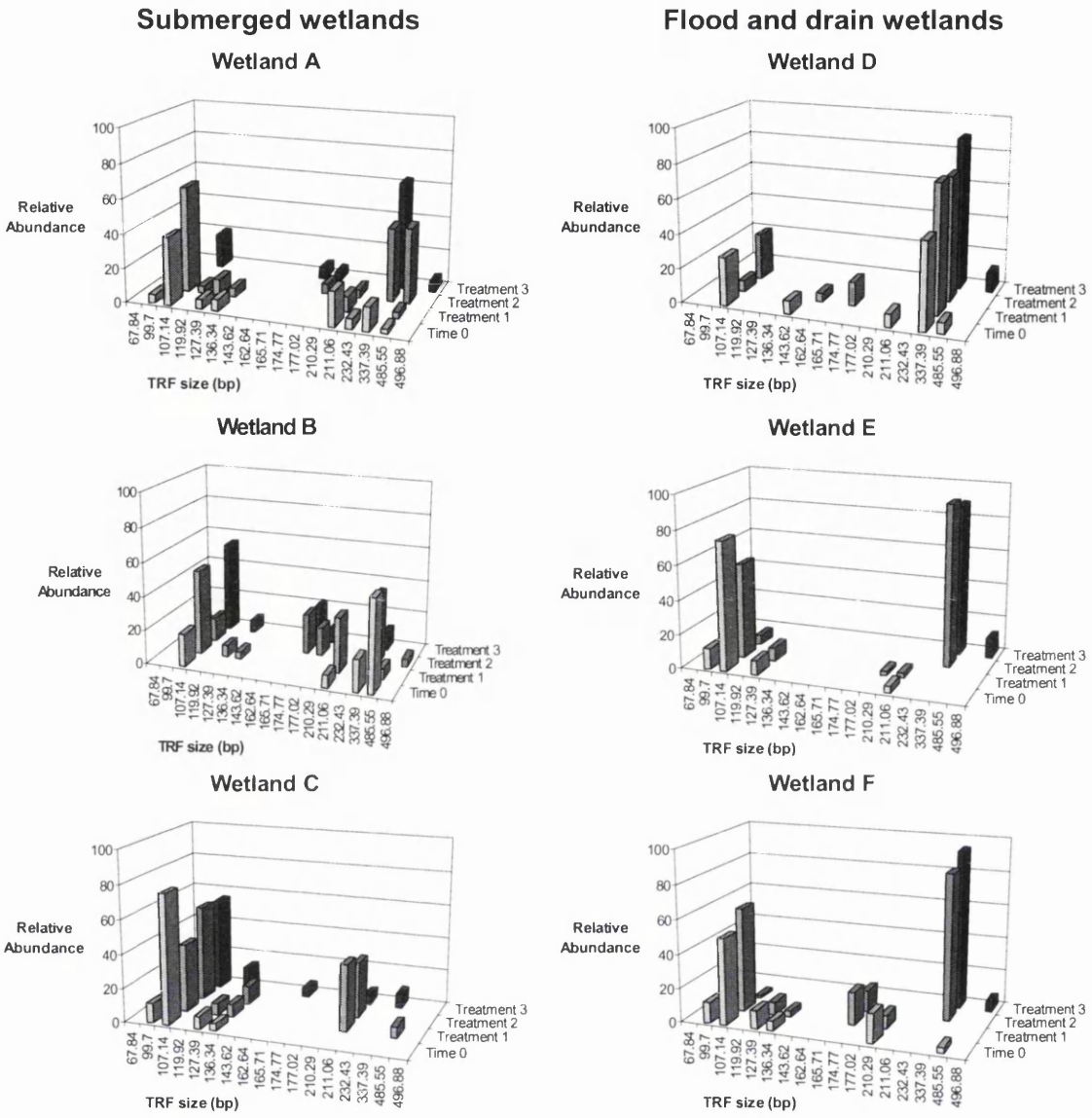


Figure 4.6. Ammonia oxidizing bacterial community structures for each wetland in different treatments. Graphs show the relative abundance of TRFs detected by T-RFLP of *amoA* gene

4.3 Summary

When laboratory scale wetlands with a single flood/drain cycle were compared to submerged wetlands, no significant differences in outlet concentrations of inorganic and organic nitrogen were observed. When

multiple flood/drain cycles were introduced, significantly lower outlet concentrations of TKN and ammonia were recorded in treatment 2 and 3, and of organic nitrogen in treatment 3. However, the concentrations of nitrites and nitrates were lower in the outlet of submerged wetlands (significant for nitrate in treatment 2 and 3). Accumulation of nitrite and nitrate was observed in the flood/drain wetlands. The elevation of nitrate concentration in flood/drain wetlands could be prevented by shortening the drainage period from 4 hours to 15 minutes. A combination of a flood/drain wetland followed by a submerged wetland showed good nitrogen removal, and resulted in lower outlet concentrations of nitrite. The results of this experiment led to a trial investigating the use of flood/drain cycles at the Selonda UK site (see Chapter 6).

In the models, the total bacterial communities in the submerged wetlands showed a greater degree of similarity to each other than those in the flood/drain wetlands. The samples taken from the submerged wetlands and the samples taken at time 0 tended to be dominated by an ammonia oxidizing bacterial OTU with a TRF of 107bp that could not be identified by T-RFLP. Bacteria belonging to the *Nitrosomonas aestuarii/N.marina* lineage were more dominant in the flood drain wetlands. It was disappointing that it was not possible to identify the ammonia oxidizing OTU with a TRF of 107bp. In this study no concerted effort was made to produce a comprehensive clone library of the *amoA* genes found in the wetlands. Given the apparent abundance of the OTU from the T-RFLP analysis, it might be expected that a cloned *amoA* fragment matching the dominant TRF could be identified if an attempt to create even a relatively small library had been made.

Chapter Five

The development of bacterial communities in a wetland treating wastewater from a land-based marine recirculation aquaculture facility

5.1 Introduction

5.1.1 Summary of the current understanding of aquaculture wetlands: nitrogen removal and microbiology

As discussed in Chapter 1 section 1.9, there have been a number of studies investigating nitrogen removal from constructed wetlands, involving either wetlands constructed at aquaculture facilities or laboratory scale models. The success of these wetlands in terms of nitrogen removal has been highly variable, but it appears that poor nitrate or ammonia removal can be a problem (see table 1.9 and section 1.9). This highlights the importance of evaluating the performance of any new wetland. Studies have been made of bacterial communities in sediments beneath aquaculture sea cages (Bissett *et al.*, 2006; Torsvik *et al.*, 1998) and inside aquaculture systems (Foesel *et al.*, 2008; Paungfoo *et al.*, 2007), but this is the first study of total bacterial communities and ammonia oxidizing bacteria in a wetland treating aquaculture waste.

5.1.2 Study aims

The primary aim of this field study was to monitor the development of the bacterial communities (total bacterial community and ammonia oxidizing bacteria) in the Selonda UK constructed wetland (see Chapter 2 for wetland description). The design of the study allowed for changes in the microbial community over time, differences between wetlands and differences between different points within the wetland to be investigated. This work also served to obtain initial baseline data on nitrogen removal in the wetland.

5.2 Experimental design

Water chemistry measurements and samples of biofilm from the wetland media were taken on 8 occasions between the start of wetland operation in January 2006 and June 2007. The sampling dates were as follows:

01/02/06	14/11/06
23/03/06	25/01/07
31/05/06	20/04/07
07/09/06	28/06/07

At the time of the first sampling event only one wetland cell-pair was operational (designated as wetland A). Thereafter two additional cells (B and C) were in use. Wetland A also received water from the storage tank overflow pipe (see Figure 2.4). This water had not passed through a Geotube®. Water samples for water chemistry analysis were taken from the storage tank, inlet to the upper cell and outlets from the upper and lower cell. All microbial analyses were carried out on samples from the upper cell only. Two samples of biofilm were removed from each wetland, from gravel just beneath the surface in the centre of the wetland at the 4th and 10th rows of distribution pipes). Additionally 500ml water was collected from the inlet and outlet of the upper cell. Sampling positions are marked on Figure 2.4.

Each sample was given a sample name, for example, sample name 6Bin indicates the sample was collected on the 6th sampling event (25/01/07) from the inlet water to wetland B. The two samples from the wetland media are labelled 2 e.g. 6B2 (sample nearest inlet), or 5 (6B5, sample nearest outlet).

5.3 Results

5.3.1 During the first 17 months of operation the wetlands reduced the concentration of inorganic nitrogen in the wastewater

Throughout the study, concentrations of ammonia, nitrite and nitrate in water leaving the wetland were typically lower than in water leaving the holding tank. (The salinity during this period ranged from 28 to 37ppt). While it was not feasible to calculate removal percentages from single time point water chemistry measurements (due to rapid fluctuations in inlet concentration, see chapter 6), these differences in inlet and outlet concentrations suggested that ammonia concentration was reduced overall across the wetlands. In most cases the ammonia concentration in water leaving the wetland was less than 0.84ppm (Figure 5.1). A notable exception was wetland A on 28/06/07 when the outlet ammonia concentration was more than twice as high as the inlet to the upper cell, by contrast the outlet concentrations for wetlands B and C on the same occasion were much lower than inlet. This difference in outlet ammonia concentrations between wetland A and the adjacent two wetlands may have indicated the start of ammonia accumulation arising from supply of unfiltered overflow water, that was observed in the flood/drain trial carried out in August 2007 (see Chapter 6).

Typically nitrate concentration in water leaving the wetland was lower than the water leaving the holding tank, notable exceptions were on 01/02/06 and 14/11/06 and 20/04/07 when nitrate concentration in water leaving the wetland was higher than water entering it. The Geotube® appeared to be an important site for nitrate removal, particularly for samples taken between

20/03/06 to 07/09/06. During the period of study outlet nitrate concentration was 0-28ppm

There was no consistent pattern of nitrite change, generally water leaving the wetland had lower nitrite concentrations than the water entering the wetland. After 07/09/06 there was a trend for nitrite concentrations to increase within the Geotube® and upper cell. Wetland outlet nitrite concentrations ranged from 0-0.54ppm.

5.3.2 No patterns in temporal or spatial changes could be detected in the total bacterial community

Visual inspection of the T-RFLP profiles (Figures 5.2a-5.2d) did not reveal any clear changes in the total bacterial community either over time or between different positions in the wetland cells. A cluster of TRFs between 69-73bp was persistent throughout the study, and often contained the most abundant fragment. Fragments of 235–240bp and 249–258bp were also persistent and on occasions contained the most abundant TRF in the samples. Further analysis by principal component analysis of the relative abundance data (Figure 5.3), and of presence/absence data (not shown) did not reveal any temporal or spatial clustering patterns. In terms of the number of OTUs detected, the minimum number of TRFs in each wetland cell was in the 07/09/06 samples and the maximum was in 25/01/07 for wetland B and 28/06/07 for the other wetlands (Figure 5.4). Considering

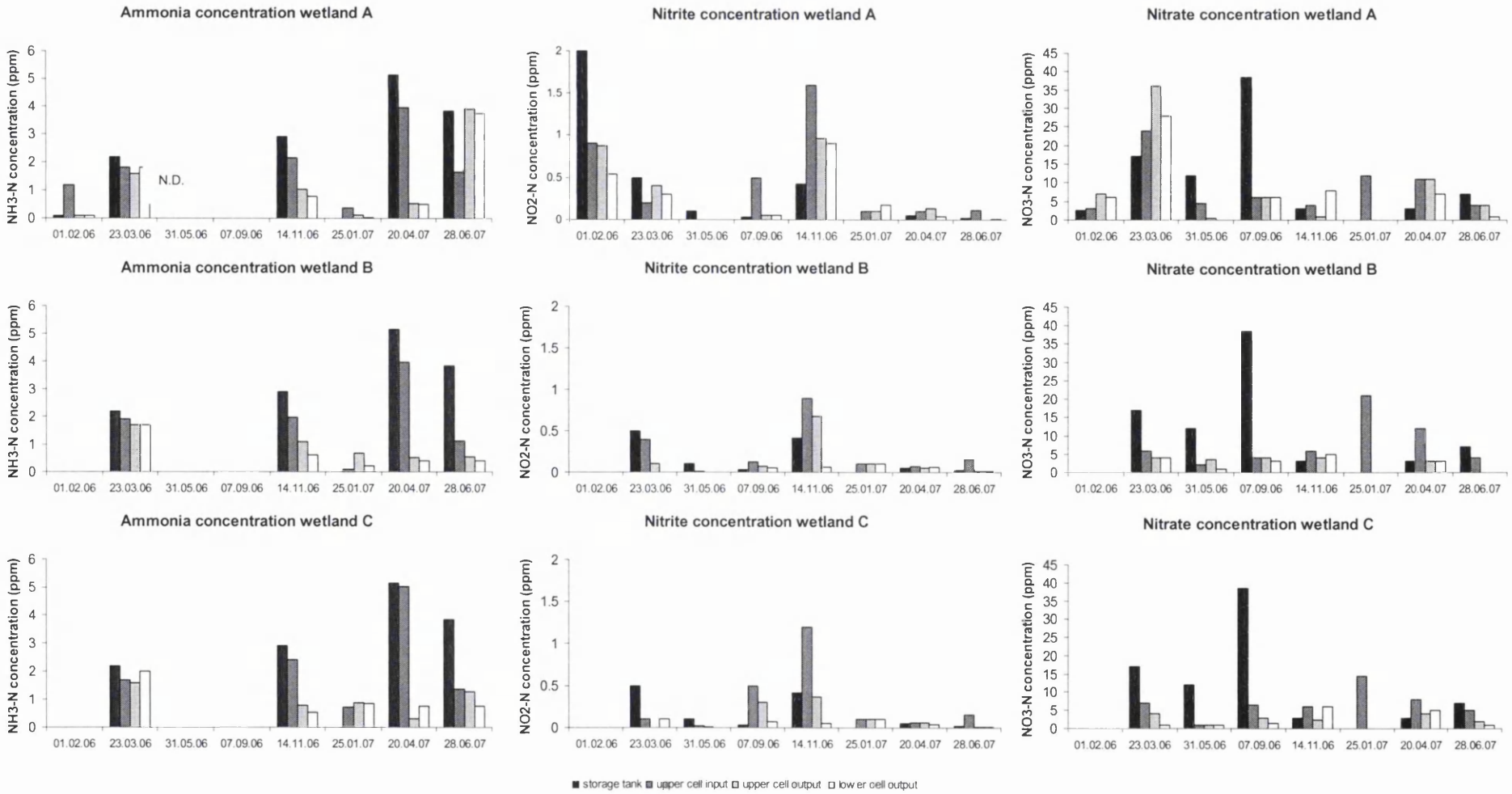


Figure 5.1. Water chemistry measurements for inorganic nitrogen concentrations throughout the period of study in the three pairs of wetland cell used in this study. Graphs show ammonia (column 1), nitrite (column 2) and nitrate (column 3) concentrations in wetlands A (row 1), B (row 2) and C (row 3). (N.D. = no data)

all three wetlands together, the number of OTUs was significantly lower in samples from 07/09/06 than all samples from 31/05/06 onwards, and lower in samples from 23/03/06 than on 28/06/07 (Mann-Whitney U test $p<0.01$, except between 07/09/06 and 31/05/06 and 14/11/06 $p<0.05$. (p values can be view in the appendix Table A4). For the whole sampling period, the number of OTUs detected in the samples taken from the wetland media was higher than in the inlet and outlet water samples (Figure 5.5). This difference was significant only between the inlet sampling point and both of the sampling points within the wetland (Mann-Whitney U test $p<0.01$).

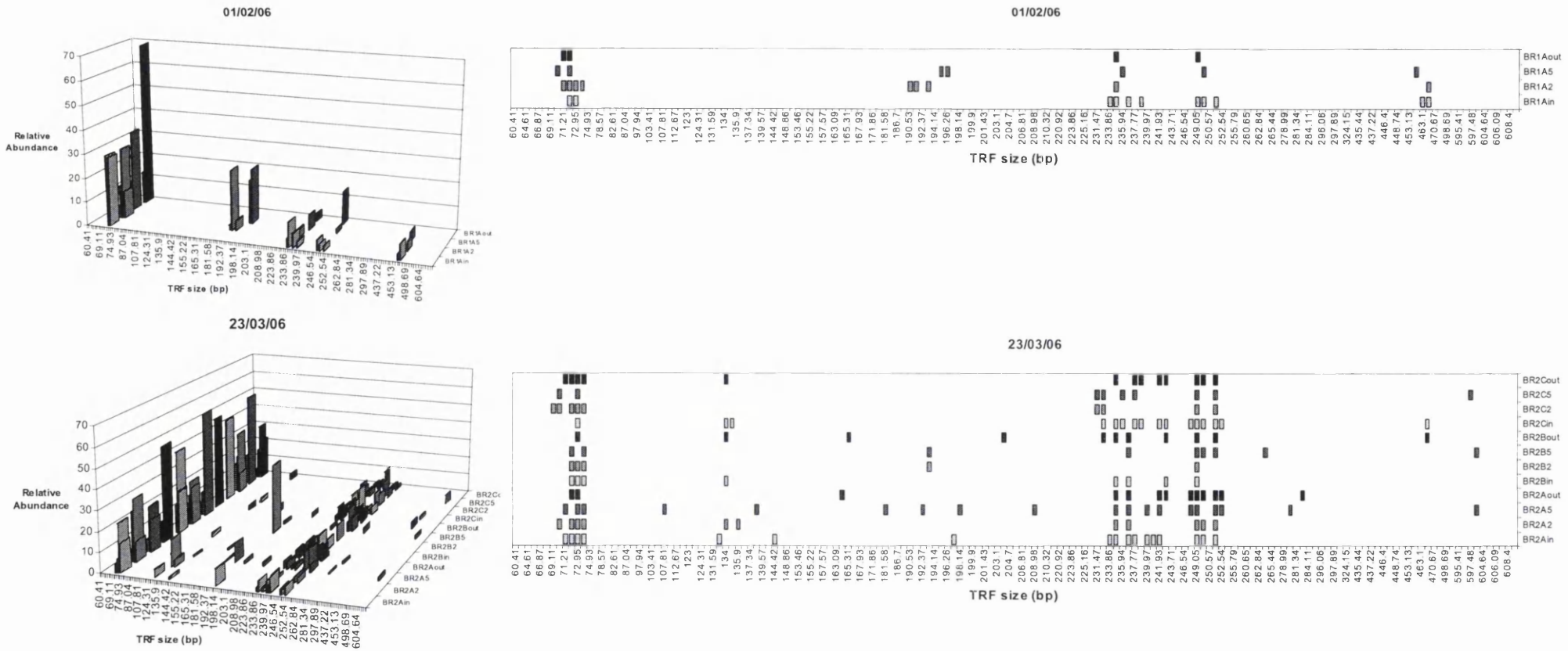


Figure 5.2a T-RFLP profiles of total bacterial community. 01/02/06 and 23/03/06. Each 3D graph (on left) shows relative abundance of different terminal restriction fragments across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of the TRFs (i.e. the 3D graphs as if viewed from directly above. See section 5.1.3 for explanation of sample coding

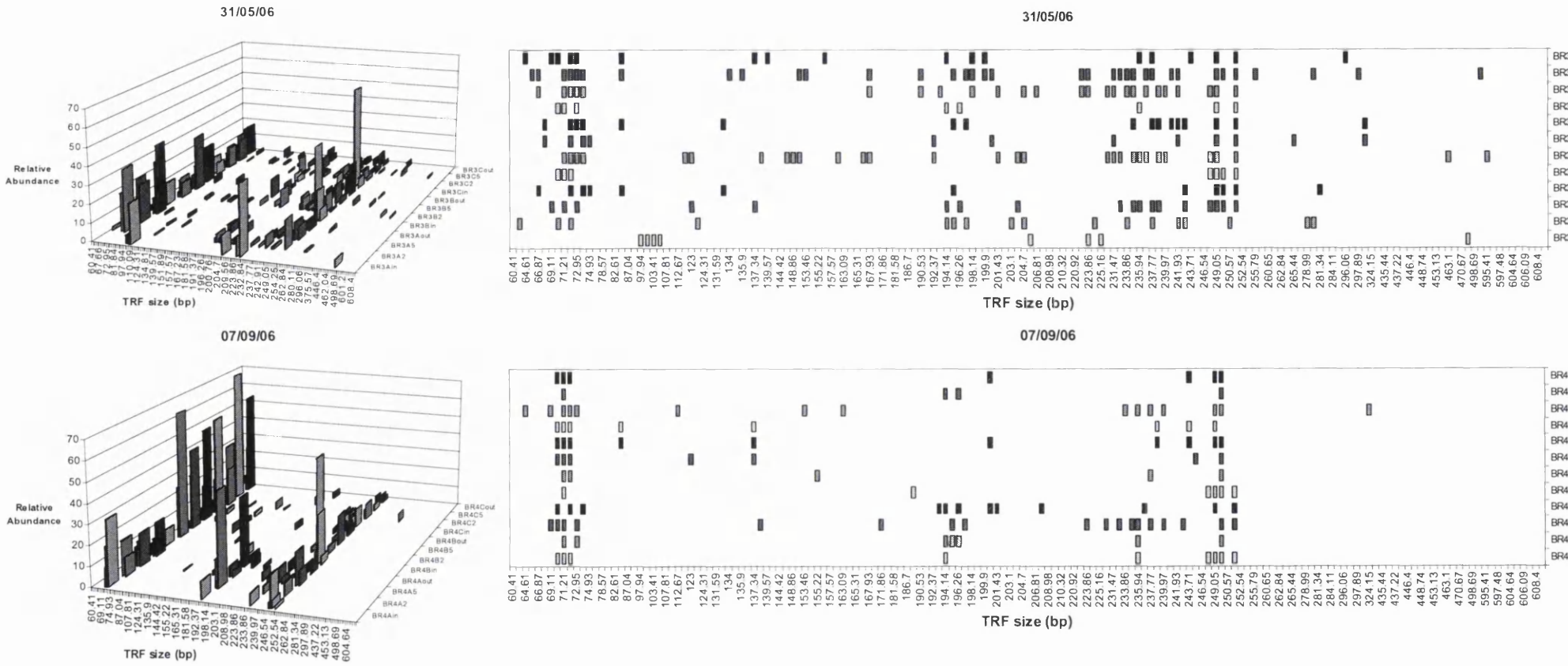


Figure 5.2b T-RFLP profiles of total bacterial community. 31/05/06 – 07/09/06. Each 3D graph (on left) shows relative abundance of different terminal restriction fragments across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of the TRFs (i.e. the 3D graphs as if viewed from directly above. See section 5.1.3 for explanation of sample coding

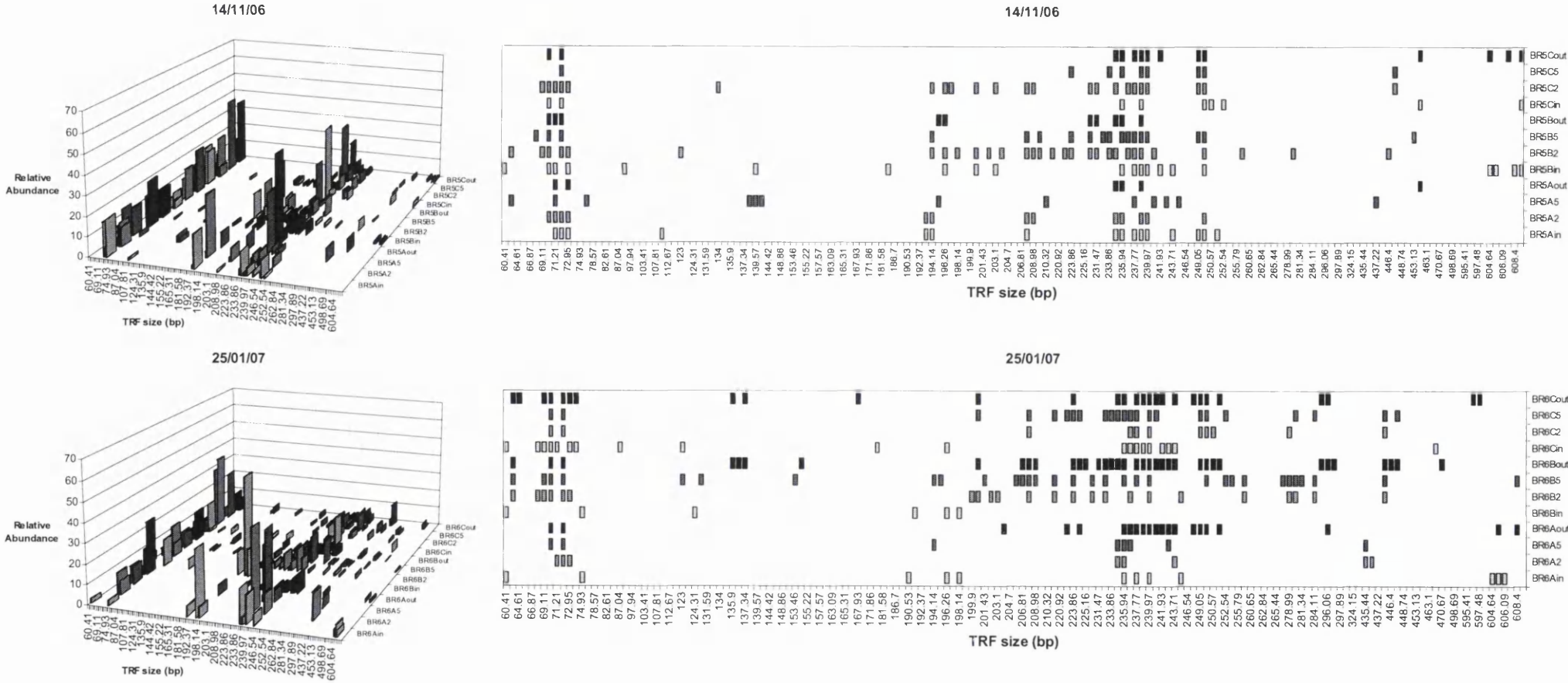


Figure 5.2c T-RFLP profiles of total bacterial community. 14/11/06 and 25/01/07. Each 3D graph (on left) shows relative abundance of different terminal restriction fragments across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of the TRFs (i.e. the 3D graphs as if viewed from directly above. See section 5.1.3 for explanation of sample coding

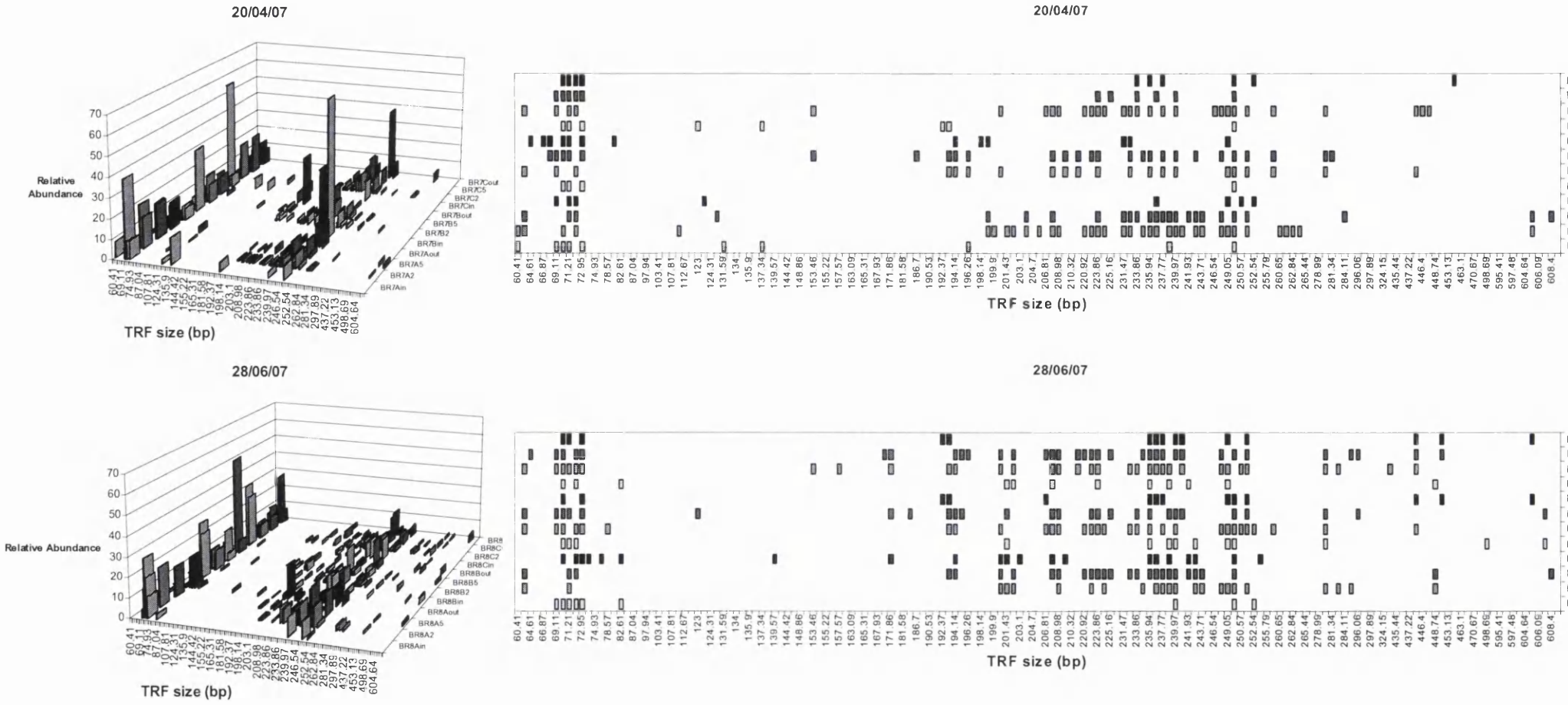


Figure 5.2d T-RFLP profiles of total bacterial community. 24/04/07 and 28/06/07. Each 3D graph (on left) shows relative abundance of different terminal restriction fragments across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of the TRFs (i.e. the 3D graphs as if viewed from directly above. See section 5.1.3 for explanation of sample coding.

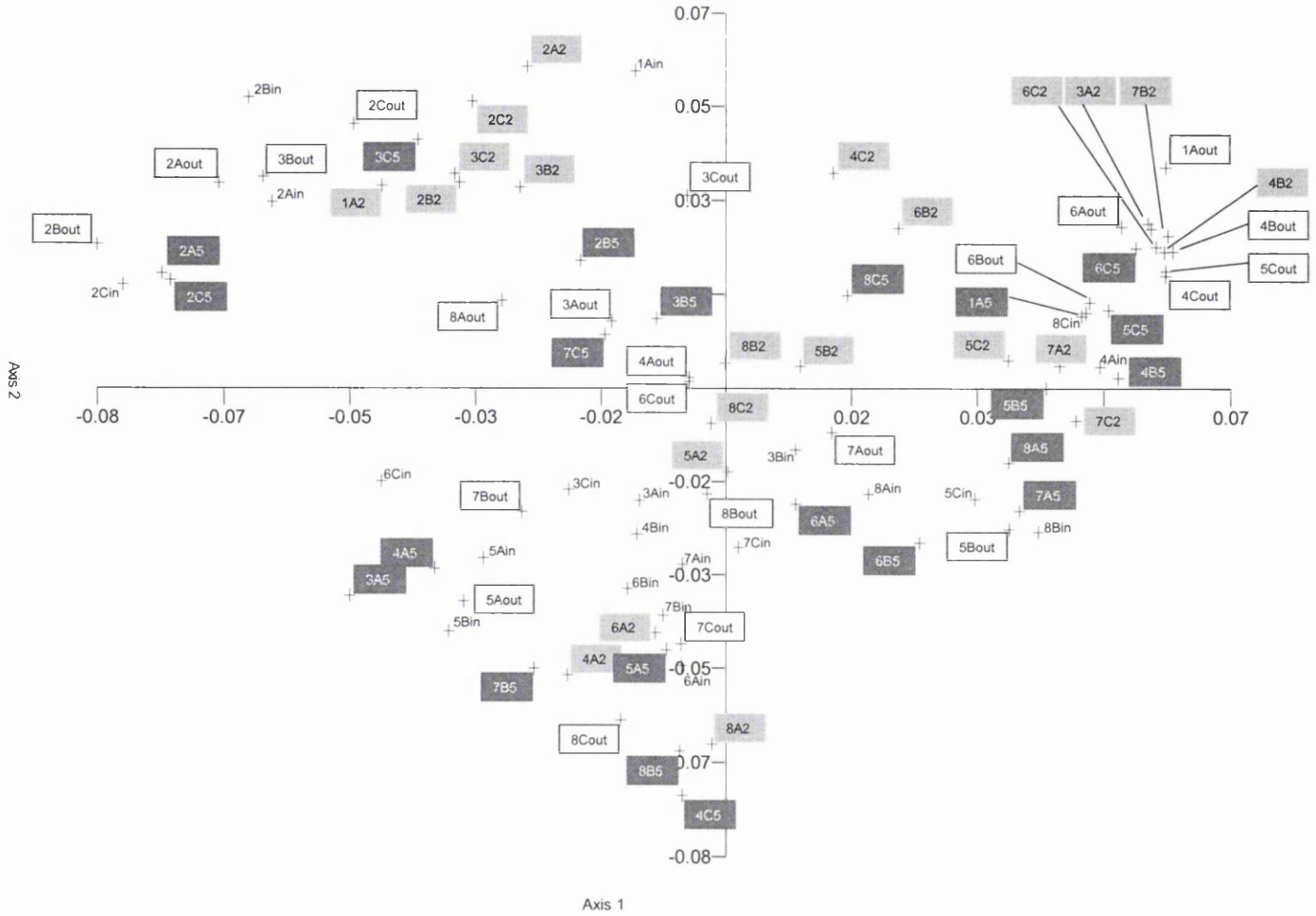


Figure 5.3. Principal component analysis for total bacterial community. Data labels shaded by sampling position Unshaded labels=inlet water samples, Light grey labels=sampling point 2, Dark grey=sampling point 5, white squares=outlet water samples. See Section 5.1.3 for coding labels.

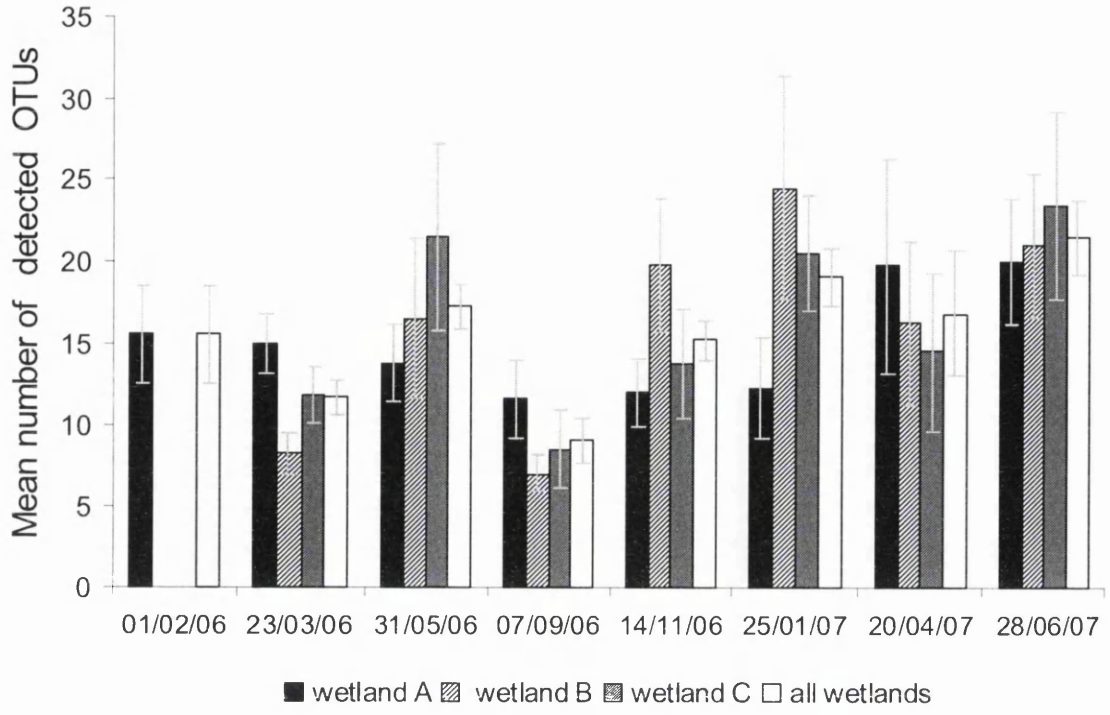


Figure 5.4. Mean number of detected bacterial OTUs in wetlands by sampling date. (N.B. Only wetland A was sampled on 10/02/06.) Error bars =± 1 SE

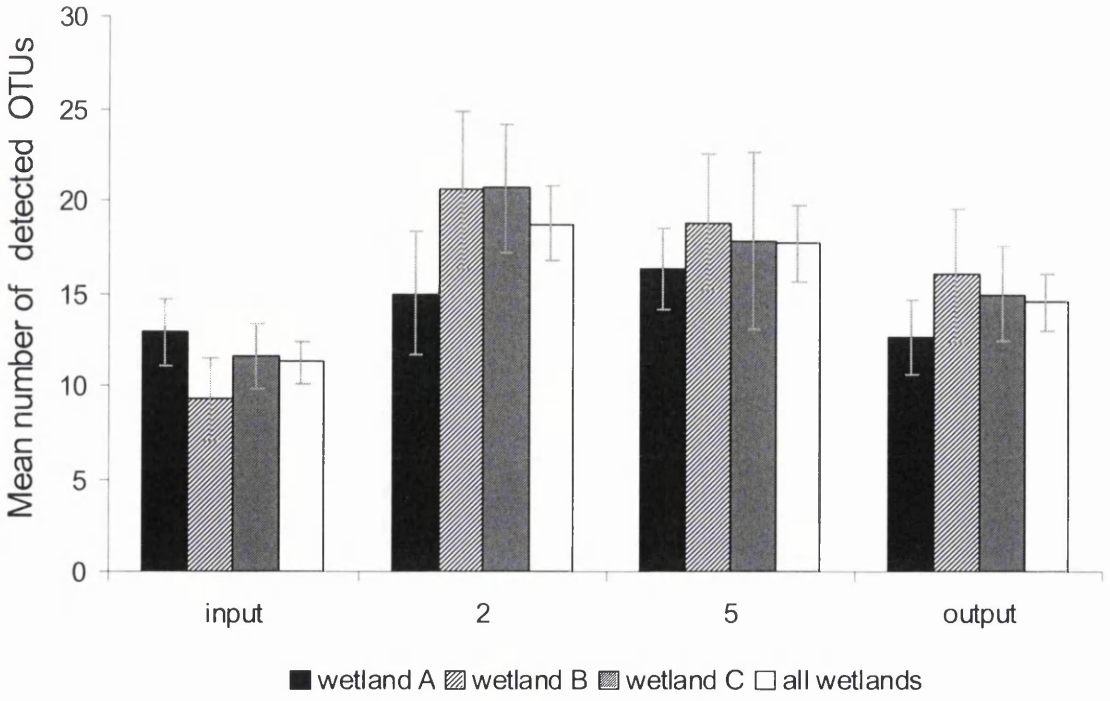


Figure 5.5. Mean number of bacterial OTUs in wetlands by wetland position. See section 5.1.3 for explanation of sampling points on x- axis. Error bars =± 1 SE

5.3.3 The ammonia oxidizing community was characterized by the persistence of one dominant TRF, with the several other TRFs being important on specific sampling dates

The most striking feature of the ammonia oxidizing bacterial community was the persistence of a 337bp TRF (previously identified as *Nitrosomonas marina/ N.aestuarii*) as a prominent component of the community throughout the study (Figure 5.6a and 5.6b). 6 out of 9 cloned bacterial *amoA* PCR products from samples collected from the wetland on 01/02/06 and 23/03/06 showed close homology with the *Nitrosomonas marina/ N.aestuarii* lineage (Figure 3.6). In total 88 biofilm samples were taken over the 17 month period, of these 73 yielded sufficient PCR product for analysis. The 337bp was detected in all but 4 of the analysed samples (4C5, 5C5, 7Aout and 8Aout). This TRF had the highest relative abundance in 42 of the 69 samples where it was present. In several samples (4Ain, 4Bin, 6A2, 6Aout, 8B2, 8B5) it was the only detectable TRF.

The second most commonly found TRF was a 107bp fragment (previously detected as an unidentifiable, but major component of submerged model wetlands in chapter 4), which was detected in 44 of the samples (most abundant in 12). This OTU was particularly common in the May 2006 samples, when it was present in all 12 samples and dominant in half of them, the April 2007 samples (present in 9 out of 10 dominant in 3) and in the June 2007 (present in 8 out of 11 samples dominant in 3). No cloned PCR products had a TRF corresponding to this OTU. However this could be because the sequenced samples were all taken at the first two sampling dates. On those dates the 107 bp fragment was only detected in wetland

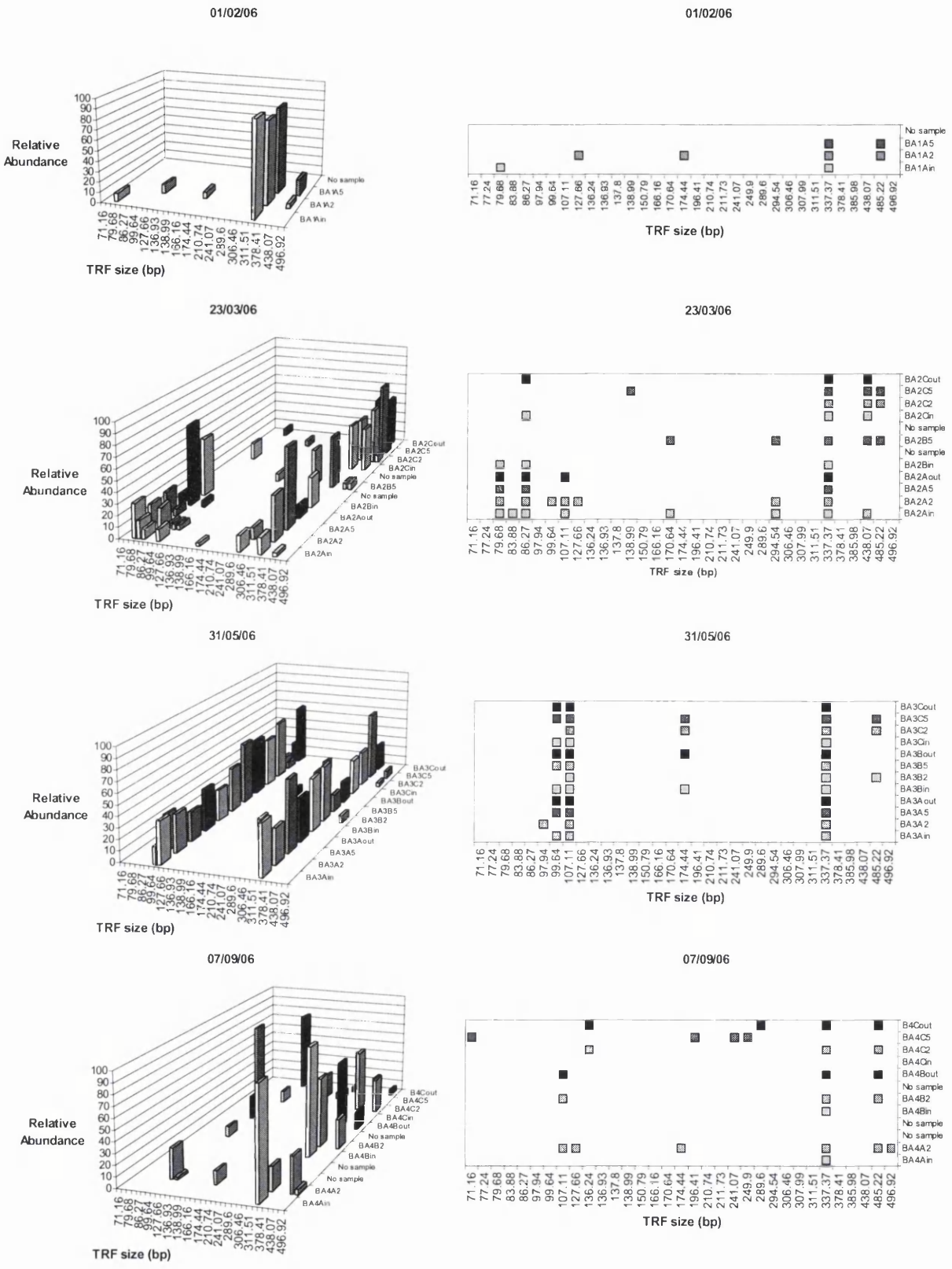


Figure 5.6a T-RFLP profiles of ammonia oxidizing community. 01/02/06 – 07/09/06. 3D graphs (on left) show relative abundance of *amoA* TRFs across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of TRFs. See section 5.1.3 for explanation of sample coding.

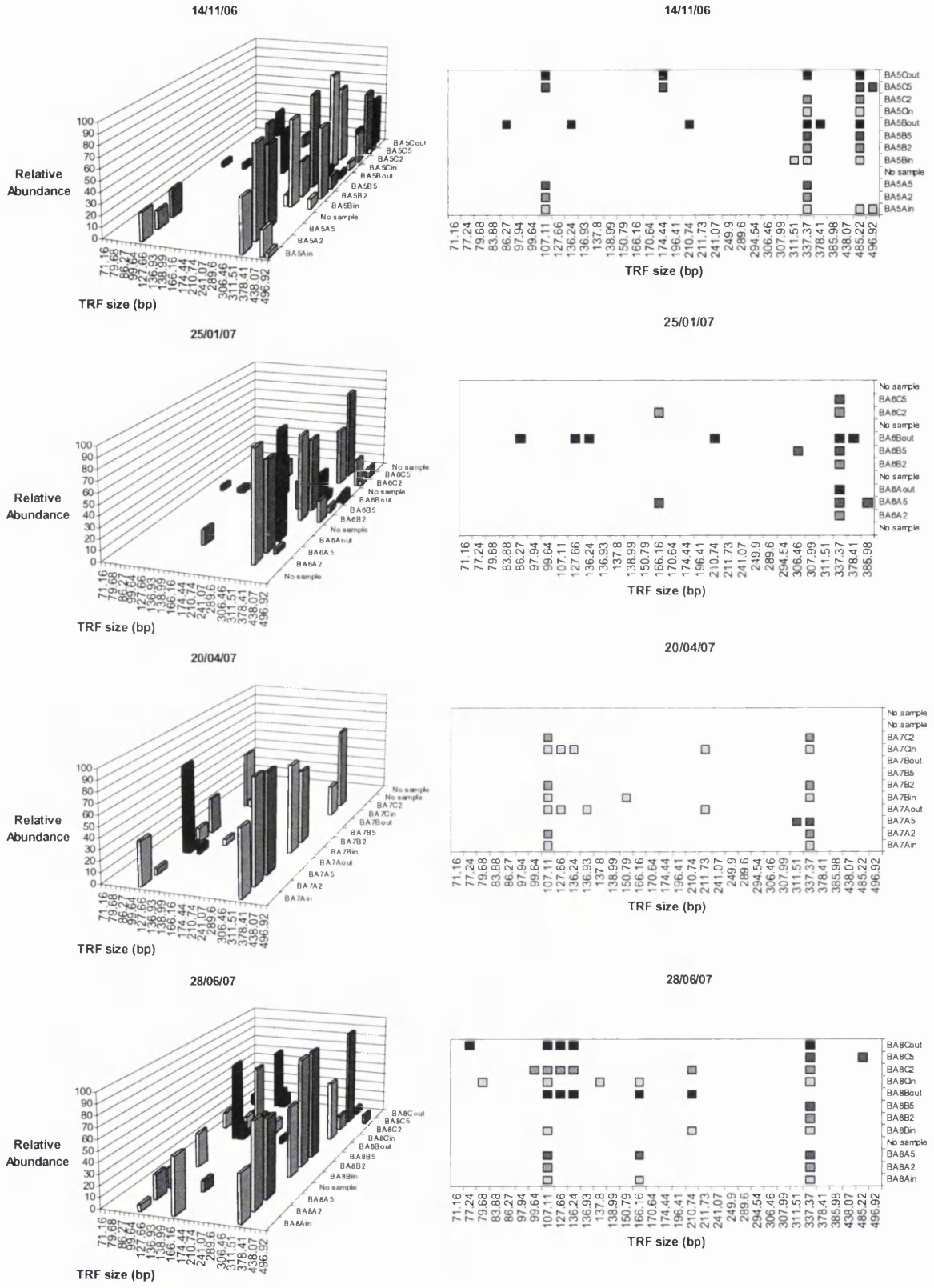


Figure 5.6a T-RFLP profiles of ammonia oxidizing community. 01/02/06 – 07/09/06. 3D graphs (on left) show relative abundance of *amoA* TRFs across the three wetlands at a single time point. The graph on the right shows the same data represented as presence/absence of TRFs. See section 5.1.3 for explanation of sample coding.

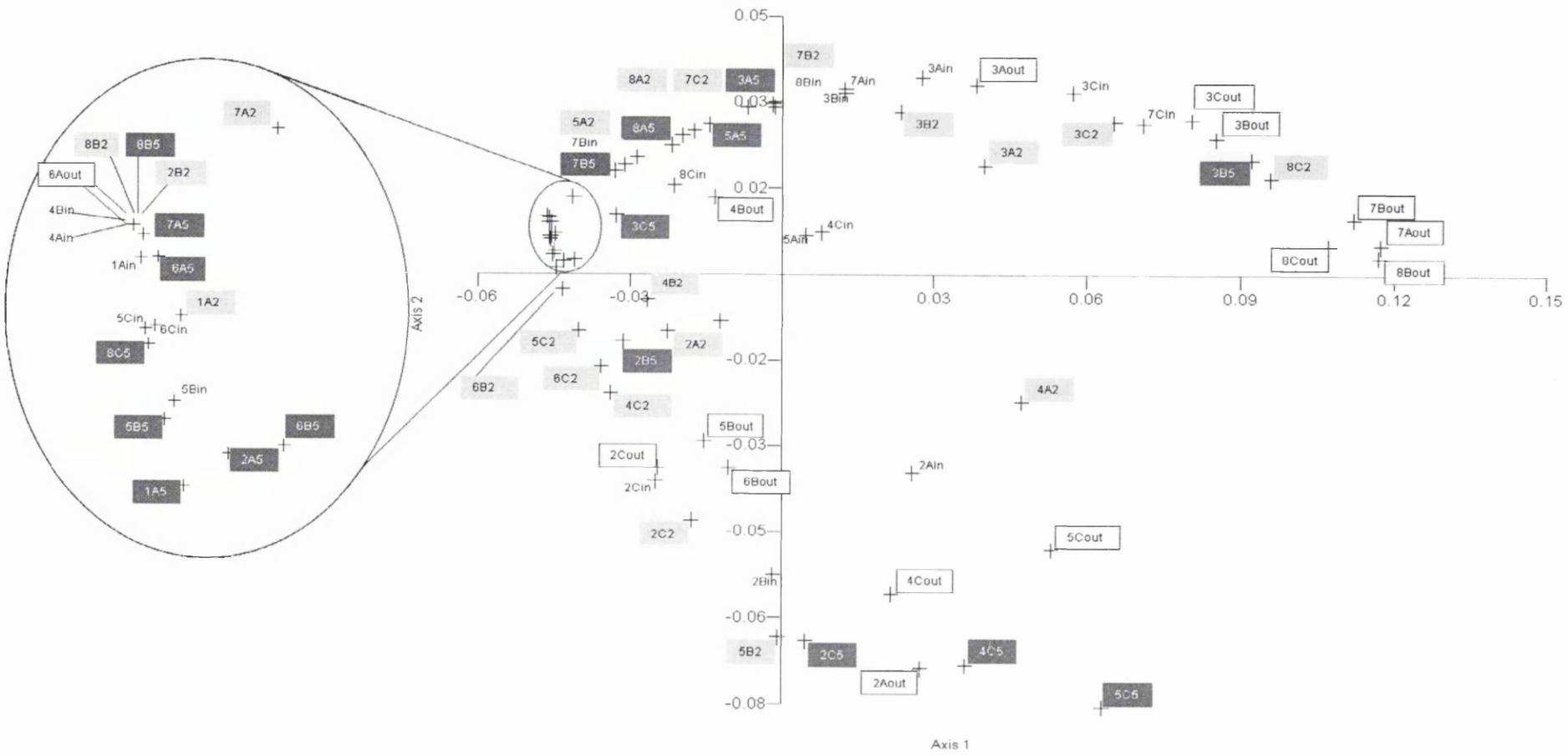


Figure 5.7 principal component analysis of ammonia oxidizing community. Data labels shaded by sampling position. Unshaded labels=inlet water samples, Light grey labels=sampling point 2, Dark grey=sampling point 5, white squares=outlet water samples. See Section 5.1.3 for coding labels

A on 23/03/06 and only 1 of the 9 sequenced clones originated from the this wetland

The third most commonly found TRF was a 485bp fragment. 2 cloned PCR products had a correspondingly sized TRF. These sequences were identified as belonging to the *N.oligotropha/N.ureae* lineage. This was detected in 30 of the 73 samples, particularly notable in November 2006, when it was observed in 10 out of 11 samples and the dominant fragment in 6 of those. Several other TRFs appeared in the wetlands and often a particular fragment appeared to be associated with a particular sampling event, for example 8 out of 11 of the 86bp TRFs occurred in the March 2006 samples, 9 out of 11 of the 100bp TRFs appeared in May 2006, and a 438bp TRFs only appears in March 2006 samples. Further exploration of the data by principal component analysis of the relative abundance data (figure 5.7), or of presence/absence data (not shown) did not reveal distinct ammonia oxidizing communities to be associated with specific positions in the wetland or specific sampling events.

5.4 Summary

Throughout the first 17 months of operation, the Selonda UK wetlands were generally effective at removing nitrogen from the wastewater, although one wetland (A) showed signs of increased ammonia concentration towards the end of the sampling period. Due to the frequency of the sampling visits, intensive monitoring of fluctuations of water chemistry of the inlet water was

not carried out until the flood/drain trial in August 2008 (see Chapter 6). When this was investigated, quite large fluctuations in inlet nutrient concentrations were observed. This indicated that reliable calculations of removal during the first 17 months of the study could not be obtained and the performance of the wetland was therefore assessed by the concentrations of ammonia, nitrite and nitrate in the outlet water. In order to obtain better measurements of removal rates, it was decided on the basis of the current findings to switch to time averaged samples for the subsequent full scale flood/drain study, in order to lessen the effect of any fluctuations.

T-RFLP and principal component analysis of the 16s rRNA gene could not detect any clear temporal or spatial changes in the total bacterial community composition, although there was a trend for increasing number of OTUs after 07/09/06. Throughout the monitoring period the ammonia oxidizing community was most commonly dominated by an OTU assigned to the *N.marina/N.aestuarii* lineage. Other OTUs such as the unidentified 107bp TRF, and one assigned to the *N.oligotropha/N.ureae* were more common on particular sampling occasions leading to the suggestion that there may be a seasonal influence on the composition of the ammonia oxidizing community.

Chapter Six

Nitrogen removal in full scale saline aquaculture wetlands: The effects of aeration by wetland cell drainage

6.1 Introduction

6.1.1 The basis for this study

A preceding laboratory study (Chapter 4) using model saline wetlands to treat aquaculture effluent water had indicated that wetland aeration by the introduction of flood/drain cycles might improve nitrification capacity, but that implementing such a regime could adversely affect denitrification. Recirculating water through the flood/drain cells was shown to be the most beneficial in the model system, however due to the difficulty of setting up this mode of operation at full scale with available resources, the effects of flood/drain versus submerged operation were tested in a single passage mode (i.e. without water recycling). The study was carried out using two adjacent pairs of wetlands (labelled B and C in Figure 2.4). Each wetland pair consisted of an upper cell that could be operated in either flood/drain or submerged mode, linked to a lower, permanently submerged cell (see section 2.5.4 for further details of wetland configuration). Adjacent upper cells were alternated between submerged and flood/drain operation, to test the consistency of inorganic nitrogen removal under different operating conditions, using the same influent source.

A study was also made of the effects of implementing flood/drain cycles on a pair of wetland cells (labelled A in Figure 2.4) that had been subject to additional loading with organic solids and that was experiencing elevated concentrations of ammonia compared to neighbouring wetlands receiving only

supernatant. Accumulation of ammonia has previously been reported in some constructed wetlands, and has been attributed to the accumulation and subsequent decomposition of organic material (Majer Newman *et al.*, 1999; Sartoris *et al.*, 1999). Organic matter consumes oxygen as it breaks down, potentially inhibiting the activity of aerobic nitrifying bacteria. De-oxygenation of surface waters has previously been shown to result in the accumulation of ammonia, reduction of nitrification capability and thus limit the amount of nitrogen that can be removed from wetlands (Reinhardt *et al.*, 2006). During decomposition ammonia is released from organic matter by deamination of amino acids, urea and proteins (Kadlec & Knight, 1996).

6.1.2 Study aims

Following on from data from the model wetlands (Chapter 4), which had shown that greater nitrification but less denitrification occurred in flood/drain wetlands compared to submerged wetlands, and on previous work by other authors showing improved total nitrogen removal in flood/drain freshwater wetlands, the aim of this study was to compare nitrogen removal in a full scale two-cell submerged saline wetland with a full scale two-cell saline wetland comprised of a flood/drain cell followed by a submerged cell.

6.2 Experimental design

The study was carried out over a 5 week period during August/September 2007. Initially, all measurements of ammonia, nitrate and nitrite concentration were taken while the wetland cells were permanently submerged. Submersible pumps were installed in the upper cells of each wetland pair, to allow the wetland cell to be drained. The water flow to the wetland varied according to operations at the fish farm but at the time of sampling was approximately 100 $l/m^2/day$. In order to standardise conditions and compare the performance of cells B and C, the inlets from Geotubes® B and C were connected in such a way that both cells received equal amounts of effluent from a common source. Flood/drain cells were drained overnight and then allowed to refill. Typically refilling took 32-34 hours. After 34 hours the cell was drained again. Water samples were collected from the inlet of the upper cell, the outlet of the upper cell and the outlet of the lower cell (see Figure 2.4). Initial test sampling indicated that the inlet concentration of nutrients was subject to constant fluctuations. Therefore, in order to be able to estimate nitrogen removal more accurately, time averaged water samples were collected instead of single time point measurements.

For the comparison of wetlands B and C the trial was divided into three phases. A flood/drain cycle was introduced to the upper wetland cells as follows (lower cell was always submerged):

Phase 1 Wetland B submerged, wetland C submerged

Phase 2 Wetland B submerged, wetland C flood/drain

Phase 3 Wetland B flood/drain, wetland C submerged

In the following text, a subscript $_{(sub)}$ indicates that both cells of the wetland pair were operating fully submerged, $_{(f/d)}$ indicates that the upper cell of the wetland pair was operating with flood/drain cycles and the lower cell operating fully submerged.

For wetland A (receiving suspended solids), the trial was divided into two phases. During phase 1 the wetland was permanently submerged. During phase 2 the upper cell operated in flood/drain mode.

When describing a change in nutrient concentration the following terms are used:

Entire wetland describes a change in concentration between the inlet of the upper wetland cell and the outlet of the lower wetland cell.

Upper cell describes a change in concentration between the inlet of the upper wetland cell and the outlet of the upper wetland cell.

Lower cell describes a change in concentration between the outlet of the upper wetland cell and the outlet of the lower wetland cell.

6.3. Results of introducing flood/drain cycles to full scale wetlands

6.3.1 Introduction of flood/drain cycles to functioning wetlands (B and C) reduced nitrate and nitrite removal, but did not affect ammonia removal

Median concentrations of ammonia, nitrite and nitrate in wetlands B and C are shown in Figure 6.1, full details of the statistical comparisons can be found in the appendix, Tables A5 an A6. For the majority of the experiment ammonia removal in the wetlands was good (62 - 100%), although on two days increases of ammonia were observed (Figure 6.2A). With the exception of wetland $C_{(sub)}$ in phase 1, a significant decrease in ammonia concentration was recorded over the entirety of both wetlands in all phases (Mann-Whitney U $p<0.05$, except wetland $B_{(sub)}$ in phase 1 and wetland $C_{(f/d)}$ in phase 2 $p\leq0.01$). The decrease in ammonia concentration was also significant in the upper cell of both wetlands in phase 2 (wetland B $p<0.05$, wetland C $p<0.01$).

Nitrite removal ranged from 43 to100% in wetland B and 4-100% in wetland C (Figure 6.2B). A significant decrease in nitrite concentration was seen over the entire wetland and the upper cell of both wetlands in phase 1 ($p<0.05$). An increase in nitrite in the lower cell of wetland C ($p<0.05$) was also observed in period 1. Outlet concentration was not significantly different to inlet in phase 2. In phase 3 nitrite concentration was significantly lowered over the entire of wetland $C_{(sub)}$ and in the lower cell of wetland $B_{(f/d)}$ ($p<0.05$).

The change in nitrate concentration ranged from 150% increase to 90% removal in wetland B and 100% increase to 95% removal in wetland C (Figure

6.2C). Nitrate concentration decreased significantly over the entire of both wetlands and in both upper cells during phase 1 ($p \leq 0.01$, except entire wetland $C_{(sub)}$ $p<0.05$). In phase 2 significant decreases were observed in the entire wetland and the upper cell of wetland $B_{(sub)}$ ($p<0.05$).

The concentration in wetland B was not significantly different to wetland C at the outlet of the upper or lower cells for any of the nutrients except in phase 3 when nitrite was significantly higher at the outlet of the upper and lower cells of wetland $B_{(f/d)}$ than the outlets wetland $C_{(sub)}$, and nitrate was higher in the outlet of the upper cell of wetland $B_{(f/d)}$ than the upper cell of wetland $C_{(sub)}$ (all $p<0.05$). Calculation of Pearson's correlation coefficient detected a large effect size (i.e. $r>\pm0.5$), attributable to the flood/drain condition in these three cases ($r=-0.816$ NO_2 upper wetland phase 3, $r=-0.826$ NO_3 upper wetland phase 3, $r=-0.693$ NO_2 lower wetland phase 3).

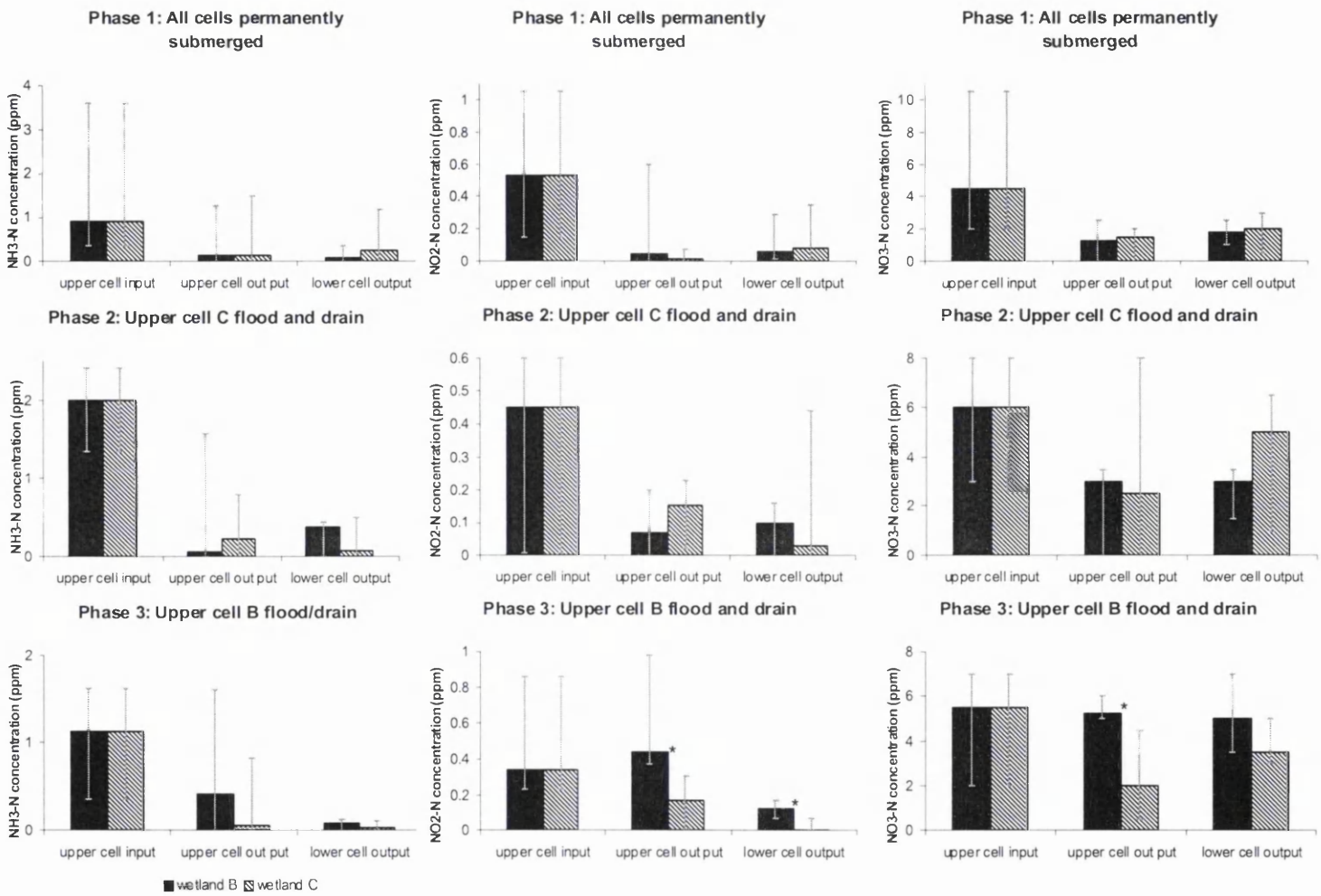


Figure 6.1. Median nutrient concentration in wetlands B and C with upper wetland under submerged and flood/drain conditions. Top row shows ammonia, nitrite and nitrate during phase 1 (both wetlands submerged), row two shows the same in phase 2 (wetland B submerged, wetland C flood/drain), and row 3 shows phase 3 (wetland B flood/drain, wetland C submerged). Significant differences are marked * for $p<0.05$ (Mann Whitney U test). Error bars show range.

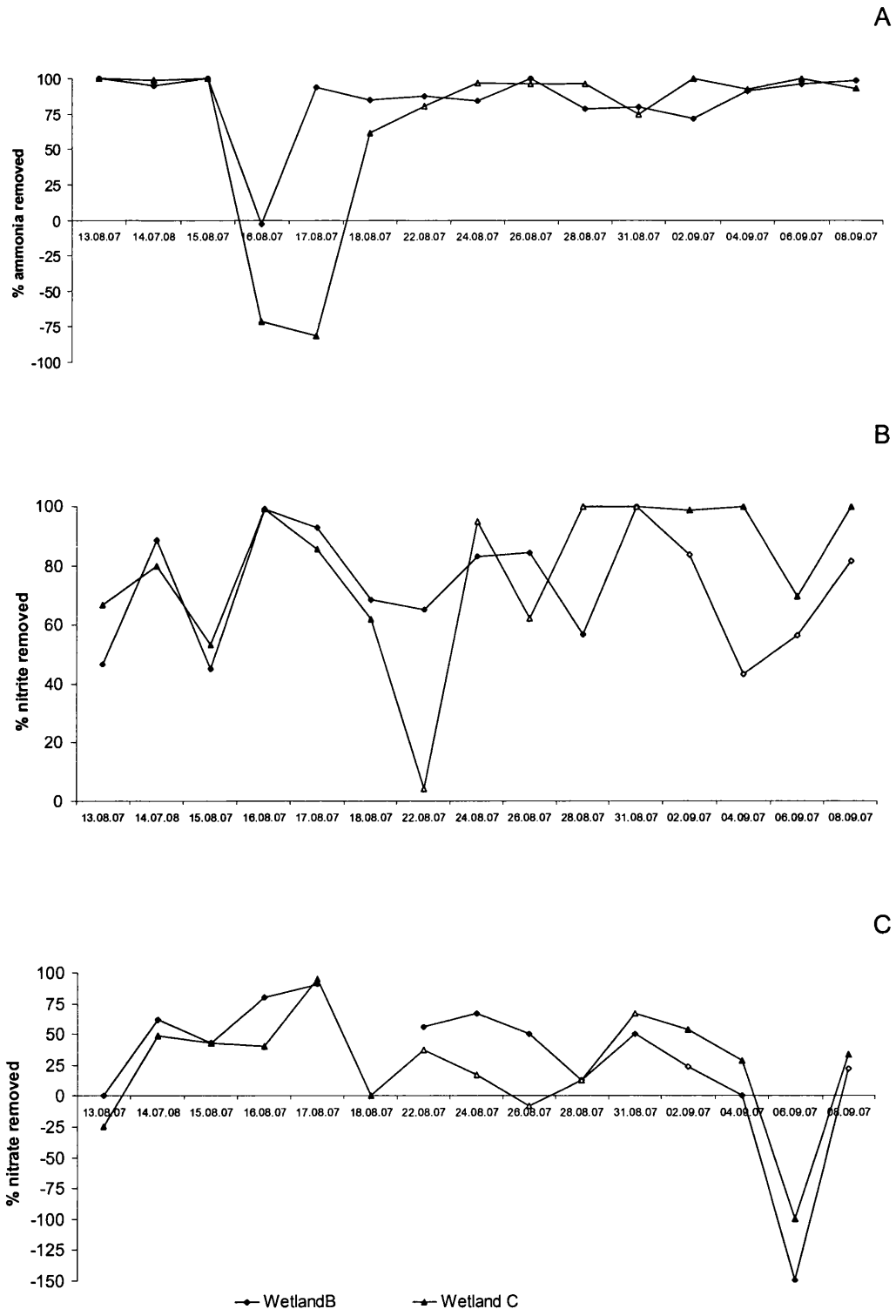


Figure 6.2. Percentage nutrient removal in wetlands B (♦) and C (▲) during flood/drain and submerged conditions. Solid symbols indicate upper cell is submerged, white (open) symbols indicate upper cell in flood/drain mode A) ammonia removal B) nitrite removal C) nitrate removal

6.3.2 Introduction of flood/drain cycles to a wetland showing elevated concentrations of ammonia rapidly restored ammonia removal

When both cells of wetland A were permanently submerged, an increase of ammonia rather than a decrease was observed in the upper and lower cells (Figure 6.3A). Significant increases in concentration were recorded over the entire wetland ($p<0.05$), and in the lower cell ($p<0.05$). When operation was switched to flood/drain mode, the ammonia concentration decreased in the wetland. The difference in concentration was significant over the entire wetland and in the upper cell ($p<0.01$). This change from increase to decrease of ammonia occurred almost immediately upon starting the flood/drain cycles (Figure. 6.4). The percentage of ammonia removed increased over time (Figure 6.5). After three flood/drain cycles the wetland consistently removed more than 73% ammonia, with the top cell alone removing at least 64% ammonia. On the final three sampling occasions (flood/drain cycles 8-10) a removal rate of over 93% was observed in the upper cell and over 95% removal from both cells combined. A maximum removal of 99.5% ammonia was observed during flood/drain period operation. Occasionally, increases of ammonia were seen in one of the cells. For example, after the first flood/drain cycle (20.08.07) an increase of 110% (1.15ppm) was seen in the lower cell (data not shown), and after the second cycle (22.08.07) an increase of 17% (0.3ppm) was seen in the upper cell (Figure 6.5). In the period when greater than 73% removal was observed there was only one occasion when ammonia increased in any of the cells; after the seventh cycle (02.09.07) ammonia increased from 0ppm to

0.37ppm in the lower cell. Although the lower cell remained permanently submerged throughout the experiment, a decrease in ammonia concentration was observed once the flood/drain cycle began in the upper cell. The reduction in ammonia concentration in the lower cell was small in absolute terms (only 0.045 - 0.605 ppm removal, reflecting the low ammonia concentration entering the lower cell due to efficient removal in the upper cell) but large in terms of percentage removal (up to 97% of the ammonia entering the lower cell was removed).

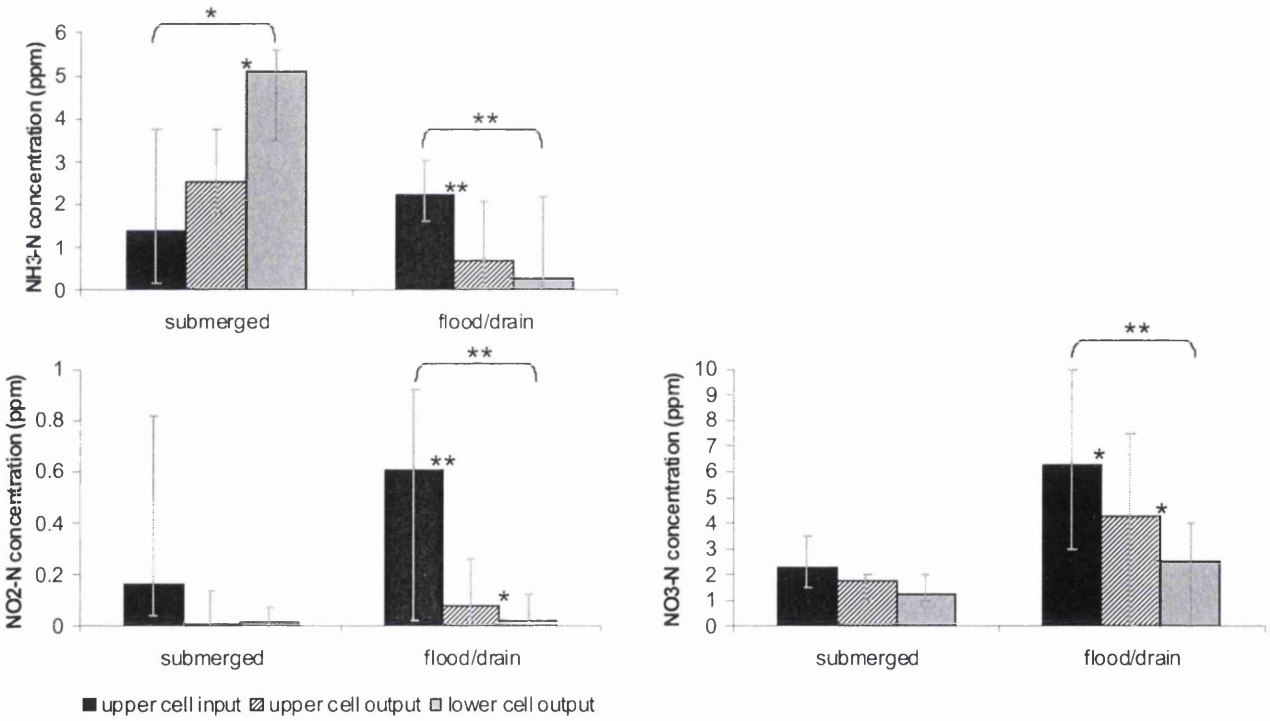


Figure 6.3. Median nutrient concentration in wetland A with upper cell under permanently submerged and flood/drain conditions. A) ammonia B) nitrite C) nitrate. Error bars show range. Significant differences between sampling points are marked * for $p<0.05$ and ** for $p<0.01$ (Mann Whitney U test). Full results from statistical analysis can be found in Table A7 of the appendix.

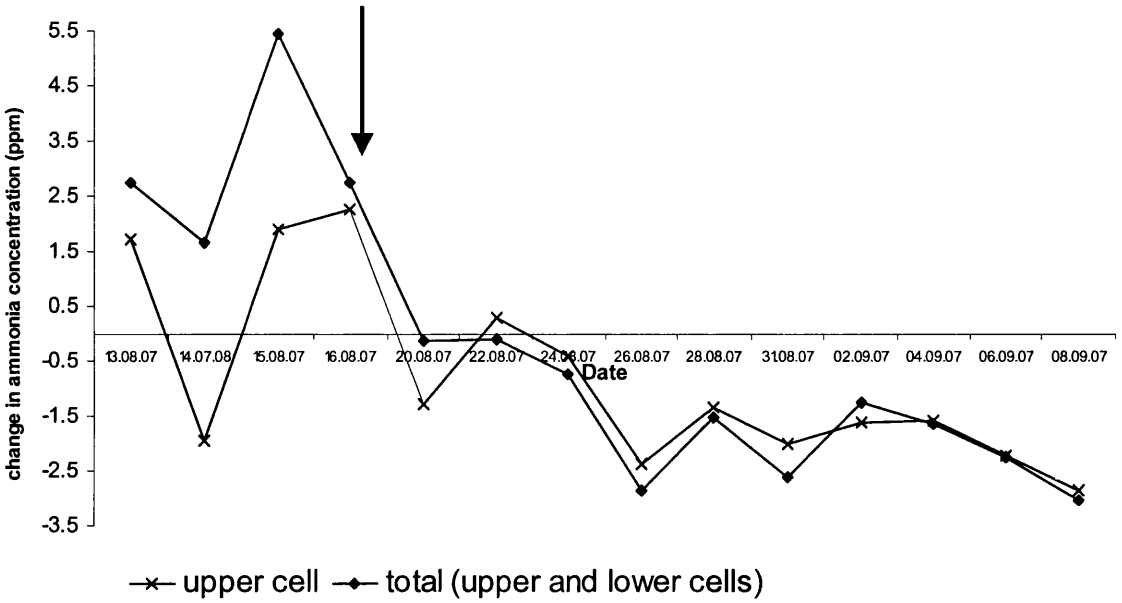


Figure 6.4. Changes in ammonia concentration observed in wetland A under submerged then flood/drain conditions. Arrow indicates point at which the upper cell was switched to flood/drain.

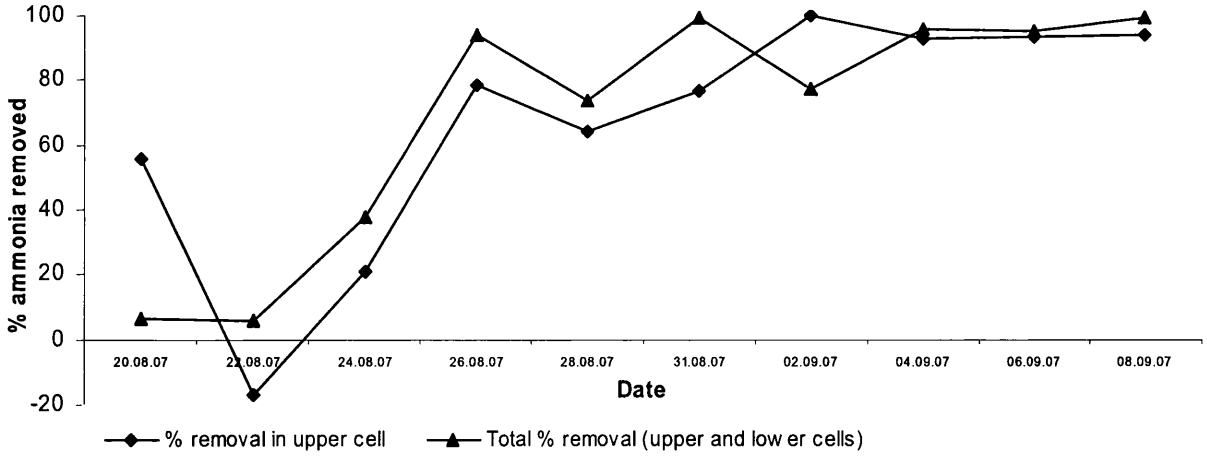


Figure 6.5. Percentage of ammonia removed from wetland A with upper cell with flood/drain cycle, lower cell submerged.

6.3.3 The introduction of flood and drain cycles improved nitrate and nitrite removal in wetland A

Throughout the experiment the nitrite removal was variable (Figure 6.6A), with no clear change being observed as a result from the switch to flood/drain cycling. At no point was the final nitrite outlet concentration higher than the inlet. A significant decrease in nitrite concentration was seen only during the flood and drain phase of the experiment (Figure 6.6B) ($p<0.05$ lower cell, $p<0.01$ upper cell and over the entire wetland). Most of the nitrite was removed in the upper cell in both submerged and flood/drain conditions.

Production of nitrate was occasionally observed in either the upper or lower cell, but there was an overall net removal of nitrate on all sampling occasions (Figure 6.6B). The observed reduction in nitrate concentration was only significant when the flood/drain cycle was operating ($p<0.01$ entire wetland, and $p<0.05$ upper cell, and lower cell).

6.3.4 Low oxygen concentrations in wetland A increased when flood and drain cycles were introduced

Dissolved oxygen was not routinely measured during this study, but readings were taken on three occasions. The first measurement was taken prior to switching any of the cells to flood/drain, the other measurements were made during the flood/drain phase. Water at the outlet of a cell had passed through the full depth of the wetland and generally had low dissolved oxygen concentrations in all cells (Figure 6.7A). In wetlands B and C the surface water

typically had higher oxygen levels than the outflow. However oxygen concentration was also low in the surface water of upper cell of wetland A. Figure 6.7B shows that after switching to flood/drain the oxygen levels in the lower cell outlet were much higher than in the submerged phase, but there was a smaller increase in dissolved oxygen concentration in the upper cell.

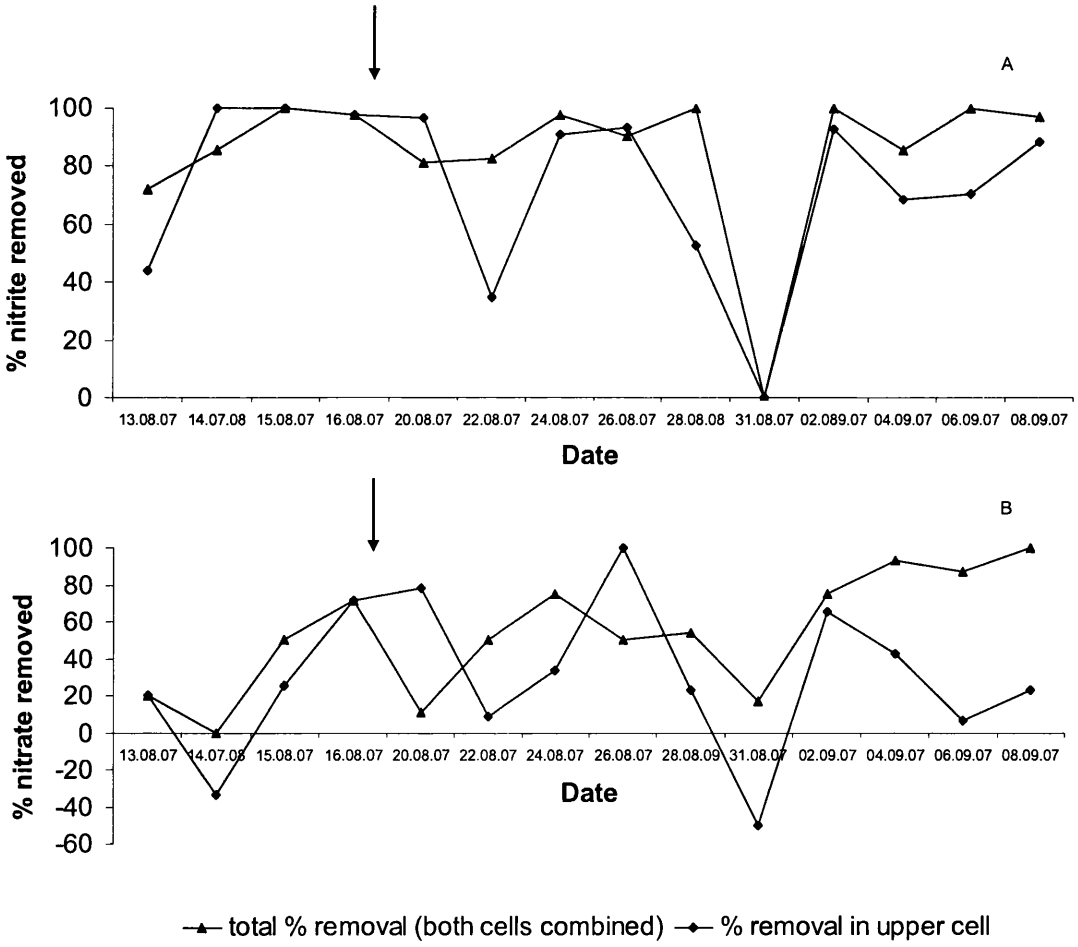


Figure 6.6. Percentage of nitrite (A) and nitrate (B) removed from wetland A with upper cell submerged and then with flood/drain cycle. Arrow indicates point at which the wetland was switched to flood/drain mode.

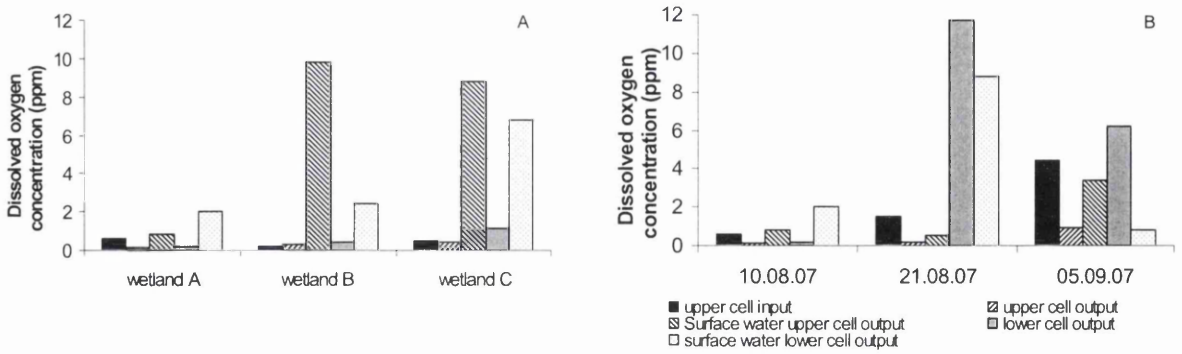


Figure 6.7. Dissolved oxygen concentration of water in wetlands A) Comparison across the three wetlands whilst in submerged operation. B) wetland A before and during flood/drain mode.

6.4 Summary

The introduction of flood/drain cycles into full scale wetlands that were showing good nitrogen removal did not improve ammonia removal, but reduced nitrate and nitrite removal, resulting in significantly higher nitrite and nitrate concentrations in the outlet of wetland $B_{(f/d)}$. When a flood/drain cycle was introduced to a wetland which had been showing a significant increase in ammonia concentration, that wetland (wetland A) began to remove ammonia. Ten flood/drain cycles were run in wetland A, and after three cycles at least 73% ammonia removal was observed and a maximum of 99.5% removal was recorded. Significant removal of nitrite and nitrate in wetland A was only observed during the flood/drain part of the experiment. This section of the study was limited in its duration. It is therefore not clear, what the long term effect of flood/drain cycles would be. Given the promising results obtained in wetland A, which had been experiencing problems with ammonia removal, longer term

monitoring is recommended in order to determine how long flood/drain cycles need to be operated in order to restore the ammonia removal function when operating in submerged mode. The benefit of flood/drain cycles in fully functioning wetlands does not appear to be substantial. Having performed this field test, the findings verified that the laboratory wetlands were a good model for the full scale wetlands. The model system therefore provides an effective tool for optimising wetland management techniques at the Selonda UK site and it is recommended that novel configurations be tested on laboratory scale before implementing in the field.

Chapter Seven

Discussion

7.1 Constructed wetlands have a capacity to remove ammonia and organic nitrogen from saline aquaculture wastewater under various conditions

7.1.1 Constructed vertical trickle flow wetlands were capable of removing ammonia from wastewater at high concentrations

The model system used to test ammonia loading capacity was capable of removing more than 97% ammonia at concentrations of up to 358ppm. A change in the nitrifying capability was identified as a consequence of increased ammonia concentration between weeks 7 and 8. Despite the percentage of ammonia removed falling at this point, the total amount of ammonia removed increased, reaching a maximum in week 9. This would suggest that the transition which occurred between weeks 7 and 8 should not be viewed as an absolute limit to the ammonia loading in this system, but as the point at which the loading became too high for the existing AOB community to adapt to immediately. As the AOB community was clearly active during period 2 (week 8 onwards) i.e. ammonia continued to be removed, it may be possible that near complete removal of ammonia from wastewater could be achieved with higher ammonia concentrations if the ammonia oxidizing community is given enough time adapt to the conditions and increase in number or activity. All three replicate wetlands received water with a concentration above 1670ppm in week 9 which is when the maximum amount of ammonia was removed. Since total ammonia removal after this time point was less, this could indicate that a concentration threshold had been crossed which adversely affected the ammonia oxidizing

community. These concentrations are well above the ammonia concentrations recorded at the Selonda UK wetlands and suggest that under the right conditions the capacity of the full scale wetland to remove ammonia far exceeds that which is likely to be necessary. Although this kind of recirculating trickle flow could be introduced into the full scale wetland, the energy costs of continual pumping are likely to result in the search for a different method of operation with a more efficient method of aeration. Methods that have been used to increase the aeration of wetlands include incorporating of passive aeration pipes to the bottom of the wetland (Brix & Arias, 2005), diffusion of compressed oxygen into the wetland (Ouellet-Plamondon *et al.*, 2006), the use of waterfalls (Monnet *et al.*, 2002) and introducing flood/drain cycles (Tanner *et al.*, 1999). The use of flood/drain cycles was further investigated in this present study.

7.1.2 Flood/drain and submerged wetlands show different nitrogen removal performances

The high nitrification capacity of the vertical trickle flow wetlands was likely to be due in part to the high availability of oxygen in this system. Aeration of the wetland by means of a flood/drain cycle offered another method of increasing the oxygen supply to the wetlands and increased the ammonia removal in the model system and in full scale wetland A at the Selonda UK site. With a single passage of water through the model wetlands (treatment 1), there was little difference between the flood/drain and submerged modes of operation for removal of any of the measured nutrients. However during treatments 2 (recirculated passage with long re-flooding

period) and 3 (recirculated passage with short re-flooding period), flood/drain wetlands were shown to exhibit better TKN, ammonia and organic nitrogen removal than the submerged wetlands. In contrast the submerged wetlands were better at nitrate and nitrite removal. This is consistent with nitrification being primarily carried out by aerobic bacteria and denitrification by anaerobic bacteria. Part of the reason for this effect only being observed when water was recirculated may simply be the residence time being lower in treatment 1, but it is likely that the successive flooded and drained periods allow for greater ion exchange and therefore greater ammonia removal.

7.1.3 Introducing flood/drain cycles to efficiently functioning full scale wetlands was not beneficial to nitrogen removal

The encouraging results from the flood/drain models prompted the introduction of flood/drain cycles into the full scale wetlands. Flood drain cycles did not improve ammonia removal from the water in wetlands that were already showing good nitrogen removal. Furthermore, as with the model wetlands, drainage cycles affected denitrification and resulted in higher outlet nitrate and nitrite concentrations than the adjacent submerged wetland during phase 3. The difference was greatest for nitrite at the outlet of the flood/drain wetland but was alleviated to some extent by passage through the submerged wetland, pointing to the benefits of using a sequential combination of flood/drain and submerged wetlands. Since these wetlands were working well at the start of the experiment, it is perhaps unsurprising that no significant improvements in ammonia removal were seen. Furthermore these findings are consistent with those from the flood/drain

model, where increases in ammonia removal were not observed when the water only had one passage through the wetland. Were it possible to configure the Selonda UK wetlands in such a way that water passed in sequence through more than one flood/drain wetland cell, it may be that an improvement in ammonia removal would be observed. Since removal of ammonia was observed throughout the sampling period, it indicates that the oxygen levels were sufficiently high to allow nitrification to proceed under permanently submerged conditions. Oxygen concentrations in the outlet water (that had passed through the depth of the wetland) were low in comparison to the surface water, suggesting that the upper layers may be critical for nitrification while denitrification can proceed in the lower layers of the wetland.

7.1.4 Introducing flood/drain cycles to a full scale wetland experiencing elevated concentrations of ammonia improved ammonia removal

When flood/drain cycles were introduced into full scale wetland A at the Selonda UK site, a rapid reduction in ammonia concentration was observed. In addition to the Geotube®-filtered wastewater, wetland A had been receiving water from an overflow pipe on the effluent holding tank. This wastewater had more organic solids than the water that had passed through the Geotubes®. It is believed that the increased ammonia concentration observed in wetland A was caused by breakdown of this organic matter. In addition to releasing ammonia this process would place a high oxygen demand on the water and create suboptimal conditions for aerobic autotrophic ammonia oxidation.

After switching the upper cell to flood/drain, both wetland cells began to remove ammonia. Measurements of dissolved oxygen indicated that drainage of the upper cell actually increased the oxygen content of the lower cell more than the upper cell. During drainage oxygen would have been drawn into the cell by the receding water, allowing aerobic respiration to occur. Since the dissolved oxygen concentration remained low in the upper cell after reflooding, a high oxygen demand must remain in the cell. The cell had a history of accumulation of organic matter (as determined by visual observation), the aerobic decomposition of which presumably consumed a considerable amount of oxygen from the water refilling the cell. Due to the physical filtering effect of the wetland the lower cell would have had a much lower amount of organic solids, so oxygen demand would be lower. It may be that the forcing of water through the pump allowed the water to become oxygenated when it escaped through the distribution pipes, which would explain the increase in oxygen concentration in the lower cell.

The observed ammonia removal seen during the flood/drain stage could be due to at least two different processes; stimulation of an aerobic ammonia oxidizing bacterial population and assimilation by heterotrophic bacteria. Autotrophic oxidizing bacteria had been detected in this wetland during the long term monitoring, and these bacteria are believed to have a number of strategies for surviving anoxic conditions, and possess the ability to recover rapidly once conditions become more suitable (Geets *et al.*, 2006). During the drained phase, when oxygen was drawn into the emptying cell the nitrifying bacteria would have been able to resume ammonia oxidation. Although increased aerobic nitrification stimulated by cell drainage may

account for some of the ammonia removal, it is also possible that aeration of the wetland could have stimulated heterotrophic growth. It may, therefore, be the case that some of the ammonia removal is due to heterotrophic assimilation rather than nitrification.

The initial failure to remove ammonia in wetland A after it had been receiving solid organic matter suggests that overloading the wetlands is a possibility at this site if a pre-treatment step is not used to filter out organic solids, and precautions should be taken against this. Since an overflow pipe will be a necessary part of the water flow control, then the use of a flood/drain cycle may be useful as a management tool to remedy any problems of ammonia accumulation.

7.2 Denitrification may be a limiting process in saline aquaculture wetlands

7.2.1 Accumulation of nitrite or nitrate could be a problem in wetlands treating aquaculture wastewater

In both of the wetland models and to a lesser extent in the flood/drain trial at the Selonda UK wetland, accumulation of nitrite and nitrate was observed. This appeared to be more of a problem in aerated wetlands, but was also observed on some occasions in permanently submerged wetlands. Accumulation of nitrate has been previously reported in other aquaculture wetlands e.g. Lin (2005), Schulz (2003 and 2004) and Sindilariu (2007). Inefficient denitrification may therefore be a particular problem in the

treatment of saline aquaculture wastewater in constructed wetlands. External carbon sources are sometimes added to wastewater treatment systems to improve denitrification, and there is evidence that carbon (either in the form of added substances, such as fructose, or carbon released from plants) can increase denitrification in wetlands (Lin *et al.*, 2002). Planting the wetlands could be a possible way to reduce the outlet concentrations of nitrite and nitrate in this wetland.

7.2.2 Elevated nitrite levels did not inhibit nitrification in vertical flow wetlands

Elevated nitrite concentrations were observed in the vertical flow wetland model in response to high ammonia loading and in flood/drain wetlands. Several authors have reported that elevated nitrite levels result from rapid increases in ammonia concentrations in various freshwater wastewater treatment plants (e.g. Burgess *et al.*, 2002; Kim *et al.*, 2006; e.g. Shiskowski & Mavinic, 1998). This accumulation may result from inhibition of the denitrification pathways or the inhibition of nitrite oxidizing bacteria by high levels of free ammonia following shock loading. In turn, elevated nitrite levels can inhibit both ammonia oxidizing bacteria and nitrite oxidizing bacteria in culture (Stein & Arp, 1998; Vadivelu *et al.*, 2006). However it has been demonstrated that ammonia oxidizing activity can be observed at high nitrite concentrations (up to 7000ppm) (Tan *et al.*, 2008). Since the highest ammonia removal in the model wetlands occurred in week 9, when the nitrite concentration was highest, it appears that concentrations of nitrite are not a limiting factor in this study and that the dominant ammonia oxidizing species in are not overly sensitive to elevated nitrite concentrations.

7.2.3 Inhibition of denitrification could explain nitrite and nitrate accumulation in model flood/drain wetlands treating aquaculture wastewater

Although nitrification in the vertical flow wetlands did not appear to adversely affected by high nitrite concentrations, elevated nitrite itself is a cause for concern and should be avoided. Results of previous studies showing that freshwater flood/drain wetlands are capable of simultaneous nitrification and denitrification were only partially confirmed for saline wetlands in this study. As with the vertical flow wetlands, nitrite accumulation was observed in flood/drain wetlands and additionally, under some treatments, nitrate accumulation was observed. This suggests that the flood/drain cycles inhibit denitrification such that production of nitrate and nitrite by nitrification is greater than their removal by denitrification. Since both the vertical trickle flow wetland and the flood/drain wetlands would have had a greater penetration of oxygen than the submerged wetlands this may be a contributing factor to the nitrite accumulation. The limit on denitrification may be the length of the flood/drain period, since nitrate reduction was observed when the drain period was reduced from four hours to 15 minutes, suggesting that longer aerated drain periods are detrimental to the nitrate reducing denitrifying populations. However, this observation should be treated with some caution as the submerged wetlands were operating under nearly identical conditions (only the displacing time was changed, (see Chapter 1 Table 1) yet significant removal of nitrate was only observed in treatment 3. Since no loss of bacterial species was observed concurrent with the disruption to nitrate removal in treatment 2 flood/drain wetlands, the difference may be due to reduced activity rather than presence and absence

of particular bacteria. Zhao *et al.* (2004) found that relatively long drained periods in freshwater wetlands improved the removal of ammonia without significant nitrite or nitrate accumulation. The present study indicated that this type of flood/drain saline aquaculture wetland might be susceptible to accumulation of both. In addition to differences in salinity, any comparison between the present study and Zhao et al (2004) should acknowledge the current use of a limestone gravel media, rather than planted soil, which may serve to protect anaerobic denitrifying bacteria from rapid exposure to oxygen and provide additional organic carbon necessary for denitrification.

7.2.4 Nitrite and nitrate removal in full scale wetlands was reduced by flood/drain cycles

When the full scale wetlands were subjected to flood and drain cycles, similar problems with nitrite and nitrate removal were observed, but only in one of the two flood/drain phases. It would appear that a drainage cycle introduced to efficiently functioning cells inhibits denitrification. It is interesting that in the case of wetland A, aerating the cell did not harm denitrification, as was seen previously in the model wetlands and in the full scale wetlands. In fact, in wetland A nitrate and nitrate concentrations were significantly reduced only when the flood/drain was in operation. This could be because the higher organic content in this wetland exerted a high biological oxygen demand and thus maintained sufficient anaerobic conditions for denitrification to proceed. Presumably the organic solids observed in this wetland in both the submerged and flood/drain phases provided a carbon source and explains why denitrification could proceed in

such circumstances. The data available from the current study do not provide a satisfactory answer as to why removal of nitrite and nitrate are significant only when the cells are aerated. Other routes of removal as well as denitrification may also be important, for example bacterial assimilation might be responsible for at least some of the nitrate removal. A potential method for investigating the contribution of these removal routes is suggested in section 7.5.1.

7.3 Ammonia oxidizing bacteria in saline aquaculture wetlands

7.3.1. Clear successional patterns of ammonia oxidizing bacteria were seen in the vertical trickle flow wetlands subject to increasing ammonia concentrations

The study on the model vertical flow wetland most clearly demonstrated the changing community structure of ammonia oxidizing species. A succession of three different AOB was observed in the wetlands, with the Nm143 lineage giving way to the *Nitrosomonas oligotropha/N.ureae* lineage at maximum ammonia concentrations and finally being replaced by the *Nitrosomonas marina/N.aestuarii.* The controlled variable in this experiment was the amount of ammonia added to the wetlands each week, and it is tempting to speculate that the changes in the ammonia oxidizing bacteria community were as a result of changing ammonia inlet concentrations. Unfortunately, in the absence of control wetlands, which did not experience the increasing ammonia concentrations, such conclusions

should not be made. However the apparently synchronous nature of the changes to the ammonia oxidizing communities in the three independent wetlands remains an intriguing observation, even if the reasons for it cannot be clearly defined. The species that dominated when ammonia concentrations were highest (week 7 and 8) was identified as belonging to the *Nitrosomonas oligotropha/N.ureae* lineage. This species is generally associated with low ammonia freshwater environments, however previous studies have suggested that different strains within this cluster are adapted for a variety of different environments. They have, for example, been identified in municipal non-saline wastewater treatment plants (Limpiyakorn *et al.*, 2005; Lydmark *et al.*, 2007). Both the *Nitrosomonas marina/aestuarii* cluster and the *Nitrosomonas* Nm143 lineage are marine species so their presence in the wetlands is not surprising. Members of both clusters have also been identified in freshwater wastewater (Urakawa *et al.*, 2006b; Wagner & Loy, 2002), and in a recirculating marine aquaculture trickling filter (Foesel *et al.*, 2008).

7.3.2 Flood/drain and submerged wetlands are dominated by different ammonia oxidizing OTUs

In flood/drain model experiments the predominant ammonia oxidizer was also influenced by wetland configuration. The development of improved nitrification or denitrification in each of the two modes of wetland operation was accompanied by a change in the ammonia oxidizing bacterial community structure. Differences in the ammonia oxidizing bacterial communities became apparent in samples taken during treatment 2 and 3 (the two

recirculated treatments with different re-flooding times). A change, marked by the replacement of the 107.14bp TRF by a 337.37bp TRF (*Nitrosomonas aestuarii/N.marina*) as the dominant OTU in the treatment 2 flood/drain wetlands, became even more pronounced in treatment 3 when all profiles had only two detectable OTUs, i.e. 337.39bp and 496.88bp fragments. In the submerged wetlands the 107.14bp TRF remained the dominant OTU. *In silico* digests revealed that three separate lineages have members that could produce a full length PCR product (496.88bp) after digestion with *Hph*I: *Nitrosomonas europaea* (of which the species *N.communis and N.halophila* are amongst those that could have produced this TRF), *N.oligotropha/N.ureae,* and the Nm143 lineage. The most similar natural environment to these wetlands would be estuarine systems (although the models and full scale wetlands in this study were subject to water with a constant salinity of about 35ppm compared to the fluctuating salinities observed in estuaries). Representatives of all three lineages have been identified in natural estuarine studies (Bernhard *et al.*, 2005; Stehr *et al.*, 1995). Since no fragments with a 497 bp TRF have been sequenced in this study a definite identity cannot be assigned. The other dominating TRF in this study, a 107.14bp fragment prevalent in all wetlands at the start of the experiment but which is replaced by the *N.aestuarii/N.marina* OTU TRF in the flood/drain wetlands, does not closely match the sizes of *in-silico* digested fragments, even taking into account the likely size shifts between observed and theoretical TRFs (Kaplan & Kitts, 2003). This may therefore represent a novel OTU that dominates in submerged saline wetlands.

7.3.3 The Nitrosomonas marina/N.aestuarii lineage dominated in the full scale wetlands at Selonda UK

The most significant OTU of ammonia oxidizing bacteria in the Selonda UK wetlands had a TRF size that corresponds to the *Nitrosomonas marina/N.aestuarii* lineage. Since the water in the wetlands had a salinity of around 35ppt it is not surprising that a species that inhabits marine environments (Koops & Pommerening-Roser, 2001) was identified as the most common species. As this OTU is common in the inlet water samples, it must populate the water prior to the wetland, perhaps originating in the Geotube® or even within the fish farm itself. It might therefore be interesting in future to examine biofilm samples from the fish rearing tanks and biofilters within the farm to assess whether changes to the ammonia oxidizing community there affect the community in the wetland. One surprising feature is the persistence of this OTU in wetland A on the final sampling event, when there was a failure of ammonia removal in that cell. It appears that this OTU is well suited to the fluctuations in environmental conditions likely to be encountered in the wetland. It would be interesting to observe whether this OTU of ammonia oxidizing bacteria would have remained the dominant OTU beyond the current sampling, while wetland A continued to accumulate organic matter and became deoxygenated.

On the basis of the model systems, finding *Nitrosomonas marina/N.aestuarii* to be the dominant OTU in the Selonda UK wetland is curious. The *Nitrosomonas marina/N.aestuarii* lineage was previously identified as being more dominant in the flood/drain wetlands in than the submerged wetlands and was the dominant OTU when ammonia

concentration was highest in the ammonia loading vertical flow experiment, so to find it as the dominant OTU in permanently submerged full scale wetlands was not expected. It may be that this TRF corresponds to a different member of the *Nitrosomonas marina/N.aestuarii* lineage which is better adapted to the wetland conditions. The 107bp TRF corresponding to the OTU found to be dominant on the submerged wetland model was also one of the most common and seasonally dominant OTU in the Selonda UK wetlands operating under similar conditions.

On the basis of the literature it is perhaps surprising that these wetlands were dominated exclusively by *Nitrosomonas* species as *Nitrosospira* species are commonly reported as the dominant species in marine environments (e.g. Bano & Hollibaugh, 2000; O'Mullan & Ward, 2005). However at least one study suggests *Nitrosospira* may dominate the water column but not the sediment (Urakawa *et al.*, 2006a) and another that in marine environments in where *Nitrosospira* species dominate the sediment samples, *Nitrosomonas* species (Nitrosomonas sp. Nm143, N.oligotropha and N.Marina) dominate the rocky biofilms (Magalhães *et al.*, 2007). The wetland substrate is a limestone gravel, so perhaps provides an environment most similar to the rocky biofilm referred to in that studies.

7.3.4 Season but not position in wetland may influence the abundance of different ammonia oxidizing OTUs

The lack of differences in bacterial community structure between different positions in full scale wetland at Selonda UK is consistent with the findings of another study which observed only limited changes in the

composition ammonia oxidizing community from media samples in different sections of a freshwater wetland wastewater treatment system (Gorra *et al.*, 2007). Elsewhere, a FISH based study of a constructed wetland found the composition of AOB communities from water samples changed during passage through a constructed wetland, but as in the present study, a group of nitrifiers (*Nitrosomonas* spp) were identified to be persistent in all samples where ammonia oxidizers could be detected (Criado & Becares, 2005).

Although different time points were not characterized by particular community structures, certain bacteria OTUs did appear to be relatively more abundant at different times, a pattern that has been observed in previous published studies. For example, Gorra *et al.* (2007) found that the abundance of different *Nitrosospira* species in a constructed wetland changed in different seasons. The results from the present study suggest that the appearance of OTUs other than the *N.marina/N.aestuarii* may be under seasonal influence. The most significant result supporting a seasonal appearance of certain OTUs is the presence of the 107bp TRF which was common in May 2006 and then became less common until April and June in 2007, suggesting an increase in this OTU in late spring and early summer. The *N.oligotropha/N.ureae* appeared in many of the November 2006 samples. Further data would be needed to confirm whether this was a seasonal effect or whether the appearance was related to other environmental variables.

7.4 Total bacterial communities in saline aquaculture wetlands

7.4.1 The largest changes to bacterial community composition in the flood/drain model were observed during treatment 1

The first treatment in the flood/drain model experiment (single passage) showed little difference between the flood/drain and submerged modes of operation for removal of any of the measured nutrients. This is unlikely to be due to a slow response of the total bacterial community at the start of the experiment due to the application of wastewater, as the T-RFLP revealed that the biggest changes to the total bacterial communities occurred between time 0 and the treatment 1 sample. The wetland media for this experiment was taken from a model wetland that had previously been used in another study, but which had been operating as a recirculating vertical flow wetland for several months with infrequent applications of fish farm effluent. It is possible that that under these low nutrient conditions a highly diverse bacterial community had developed with many species, each with low relative abundance. Within the first few weeks of this experiment the number of OTUs detected decreased in both submerged and flood/drain conditions. It can be speculated that exposure to comparatively high nutrient concentration within this experiment led to the development of communities with fewer detectable OTUs. This would be consistent with other studies on microbial communities associated with aquaculture waste, which have seen that exposure to wastewater reduces the number of bacterial species detected in sediment samples (Bissett *et al.*, 2006; Torsvik *et al.*, 1990). The T-RFLP analysis of the flood/drain model similarly only managed to pick up slight

differences between the flood/drain wetlands and the submerged wetlands. The high initial inter-sample diversity remained in the submerged wetlands throughout the experiment, but the flood/drain wetlands became more similar to one another. This might reflect a selective pressure on the bacterial population caused by the fluctuating conditions resulting in the formation of a community composed of species tolerant to a wide range of oxygen concentrations.

7.4.2 The bacterial OTUs seen to increase in the model flood/drain wetlands might be aerobic denitrifiers

In the analysis of the total bacterial communities the relative abundance of two TRFs (238.51bp and 249.38bp) increased in the flood/drain wetlands. It may be that these OTUs are suited to the fluctuating oxygen conditions of the flood/drain wetlands, and may be important to the bioremediation capabilities of the wetlands. Oxygen tolerant or aerobic denitrifiers are among the species that are known to increase in flood/drain wetlands, and in particular one study identified a 50% increase in the abundance of *Paracoccus denitrificans* (Maciolek & Austin, 2006). Although further investigation would be needed to be able to identify the two increasing TRFs, it is tempting to speculate that the 249.38bp TRF could represent *Paracoccus denitrificans*, as the predicted TRF of the sequenced strain has a size of 250bp (Paracoccus denitrificans PD1222 Genbank: CP000489).

7.4.3 No seasonal or spatial differences in bacterial community composition could be detected in the full scale Selonda UK wetlands

Examination of the total bacterial communities in the full scale wetlands did not reveal any seasonally distinct communities as determined by principal community analysis. Some changes over time in terms of OTU number were identified with an OTU minimum being identified on 07/09/06 with a trend of increasing OTU number thereafter. On the basis of experiments on the model systems, a change in the community structure might have been expected as an adaptive response to the input of effluent or changing environment. However, in the model experiments, the biggest changes in community structure were observed in relatively extreme conditions, for example very high concentrations of ammonia or the introduction of drainage cycles. Perhaps the constantly flooded mode of operation in the current field study did not force the development of specific community structures in the full scale wetlands. Previous work using both T-RFLP and a 16s rDNA clone library found no seasonal changes in a natural wetland (Kraigher *et al.*, 2006), so it may be that seasonal influence on total bacterial communities is not detectable by the methods used.

7.5 Summary, recommendations and conclusions

7.5.1 General considerations and future work

What then is the significance of this work in the wider context of understanding the microbial processes of wastewater treatment? In contrast

to much of the published molecular microbiology work, this study has examined the dynamics of communities in a marine wetland system over a 17 month period rather than simply building an inventory at a single time point. While this more comprehensive approach did not reveal any distinct or systematic changes to the bacterial community, it has indicated that community differences may be as great between different sampling points in one wetland as between different wetland cells or different time points. This indicates that to establish a comprehensive species list for a water treatment system may not be possible from a single time point and may require considerably more sampling intensity than typically used in order to get a global picture of the microbes present. This point can be illustrated by reference to the results of the ammonia oxidizing community analysis. Sampling at a single time point would have identified the 337.37bp TRF as an important OTU, but would not have necessarily have picked up the seasonal appearance of other OTU. On the other hand, the limitations of the T-RFLP approach are that it is not easily possible to obtain comprehensive species lists. To fully understand the microbial processes occurring within constructed wetlands it would be necessary to combine species identification by sequencing with community profiling by T-RFLP. Since the costs of sequencing have fallen in recent years, and with the increasing availability of high throughput technologies, such as 454 pyrosequencing, compiling a representative inventory has become a realistic possible for future studies (Hamady *et al.*, 2008). If this inventory could be combined with techniques such as T-RFLP, it may be possible to observe the dynamics of a large number of identifiable OTUs over a period of time and link these changes to

biogeochemical processes, thus improving our understanding of bacterial ecology and its influence on wastewater treatment.

This study has successfully identified changes that occur in the autotrophic ammonia oxidizing bacterial communities, but to fully understand the nitrification process future work would focus on the enumeration of AOB, and the quantification of metabolic activity, for example by the use of quantitative and RT-PCR. Furthermore, recent work has identified a potential role for ammonia oxidizing archaea and anaerobic ammonia oxidizers which should be investigated for a comprehensive understanding of this system.

This study has identified that flood/drain cycles are a useful tool for wetland management. However, continual flood/drain cycles are not necessary or desirable for efficient performance, and considering that continual pumping would incur a financial cost in terms of electricity, so it would be prudent to identify the optimal timing for flood/drain cycles. Future studies could address several questions relating to this, for example: Can imminent nitrification failure be predicted by monitoring water chemistry and elevated ammonia concentrations be avoided by implementing flood/drain wetlands? Could elevated ammonia be prevented by introducing periodic flood/drain cycles e.g. every six months? Will ammonia removal continue after the flood/drain cycles are stopped? What is the optimal number of cycles needed to restore nitrification?

The answer to these last two questions in particular will require greater understanding of the potential role of nitrogen assimilation. If nitrogen assimilation is an important process in flood/drain wetlands then presumably enhanced ammonia and/or nitrate removal would only continue while the

organic loading is sufficient to maintain high heterotrophic growth rates, and eventually nitrogen stored in biomass will be returned to the water. One approach to quantifying the relative contribution of nitrification and assimilation would be to use a stable isotope (^{15}N) approach. Laboratory studies using media from the wetland would enable assimilation of different types of labelled nitrogen (ammonia, nitrate, organic nitrogen etc.) to be quantified, as well as measuring the amount of nitrogen that is removed as gaseous products of denitrification. A major aim in microbial ecology remains linking the identity of organisms to their function. Making this link will allow greater understanding of nutrient cycling and the basic biology of the organisms involved. Additionally this information will provide knowledge that will be applicable to the optimization of industrial processes. The key to achieve this aim seems to be to combine molecular microbiology with traditional microbiology. Improving and developing novel high throughput culture methods will allow the isolation of as yet uncultivable bacteria. Once pure cultures are obtained, these can be screened to identify useful or interesting metabolic activity in isolates. When looking at environmental microbiology, techniques such as microautoradiography and fluorescent in situ hybridization (MAR-FISH) maybe able to link identity to function and has previously been used, for example, to study heterotroph-autotroph interactions (Kindaichi *et al.*, 2004) and could be applied in this area.

Another area of potential future research would be to investigate whether planting the wetlands with salt tolerant species would be beneficial. Studies have consistently shown that planted freshwater wetlands remove more nitrogen than unplanted ones, including at least two studies using

freshwater aquaculture waste (Naylor *et al.*, 2003; Ouellet-Plamondon *et al.*, 2006). For wetlands treating saline aquaculture waste, planting with species such as *Juncus kraussii* and *Suaeda estoroa* have been found to increase inorganic nitrogen removal when compared to unplanted wetlands (Brown *et al.*, 1999; Lymbery *et al.*, 2006). Any cost-benefit analysis would have to weigh up the additional cost in terms increased management demands (e.g. planting and harvesting) against the benefits of improved nutrient removal. Since the wetlands have been shown to be capable of good levels of nitrogen removal (unless additional solid waste is applied) it seems unlikely that there would an advantage to planting the wetlands unless an improvement to nitrate and nitrite removal through denitrification was desired or if the wetlands were planted with a useful or commercially valuable crop such as animal feed or oil seed crops (e.g. Brown *et al.*, 1999) or ornamental plants (e.g. Belmont *et al.*, 2004).

7.5.2 Summary of main findings on bacterial community composition

A reduction in the number of OTUs was observed concurrent with a reduction in the percentage of ammonia removed from model vertical trickle flow wetlands subject to increasing ammonia concentrations. In the same wetlands the ammonia oxidizing community changed in a way that was consistent across the three independent replicates. Whether this was by chance or as a result of the changing ammonia concentrations could not be determined due to limitations in the experimental design (i.e. wetlands which received wastewater without the additional ammonium chloride were not run in addition to the three replicates). This study has also shown that the

communities of ammonia oxidizing bacteria developing in flood/drain wetlands are distinct from those in submerged wetlands. Flood/drain wetlands and the Selonda UK wetland were dominated by an ammonia oxidizing bacteria belonging to the *Nitrosomonas aestuarii/N.marina* lineage. Submerged model wetlands were dominated by an unidentified ammonia oxidizing OTU.

7.5.3 Conclusions and recommendations for wetland operation

The work on the flood/drain wetlands established the effectiveness of using model systems to predict function in full scale wetlands and additionally provided the background information that resulted in the successful use of flood/drain cycles to re-establish nitrification in the failing wetland. Therefore model wetlands offer a valuable tool for predicting wetland performance in situations where it is impractical or undesirable to carry out experiments on the full scale wetland. For example, models could be used investigate scenarios such as the overloading the wetland with concentrated wastewater or applying the settled solid wastes to wetlands, without risking damage to the wetland or the environment. In this study the work on vertical trickle flow wetland models has demonstrated their capacity to treat saline wastewater with ammonia concentrations that are many times greater than are typically encountered in aquaculture wastewater. Although the maximum capacity was not tested in flood/drain wetlands it was shown that multiple flood/drain cycles offer improved TKN, organic nitrogen and ammonia removal, but lower nitrate and nitrite removal. In terms of total nitrogen removal, any increase in TKN removal is matched by a decrease in nitrite/nitrate removal and the total

nitrogen removal in both modes of wetland operation was remarkably similar in the first 3 treatments. The trade off between nitrification and denitrification observed in the models was reproduced in the full scale wetlands and identified that different modes of operation (flood/drain or submerged) can be used to target removal of specific nitrogen species. Total nitrogen removal in the combined wetland model (treatment 4) was lower than in treatment 3, (median removal of 62.3% compared to 81.6% and 86.6% for submerged and flood/drain wetlands respectively), but the combined wetland showed exceptional ammonia removal, and did not suffer from the considerable nitrite accumulation that was observed with treatment 2 and 3. On the basis of the model wetlands, a combined wetland system, the first operating with a flood/drain cycle followed by treatment in a submerged wetland appears to be the most suitable solution for efficient nitrogen removal combined with maintenance of low nitrite concentrations when treating of aquaculture waste in constructed wetlands. Furthermore a significant difference in the removal of nitrogen species is more likely to be observed if the water is circulated through several flood/drain or submerged wetlands. The optimal combination of flood/drain wetlands and submerged wetlands could be investigated further by use of additional flood/drain wetland experiments.

Measurements carried out at the Selonda UK wetland show that under a submerged flow management regime long term removal of nitrogen is only achieved when organic solids are removed by filtration through Geotube® filters. When overflow solid waste is applied to submerged wetlands ammonia accumulation may be observed. The introduction of flood/drain cycles into a wetland cell which was producing ammonia was shown to have

an immediate beneficial effect on nutrient removal. Within a few cycles near complete ammonia removal was observed. Denitrification was not adversely affected, and nitrite and nitrate removal also increased in the flood/drain mode.

The use of flood/drain cycles in wetlands that were functioning well was not shown to have any beneficial effect. Indeed there was evidence that denitrification could be adversely affected by the introduction of a flood/drain cycle. It is therefore recommended that flood/drain cycles be used if failure to remove ammonia is observed in a wetland, but that flood/drain cycles are not routinely used in the wetlands that are not showing accumulation of ammonia.

Appendix

Results of statistical analysis carried out for this study are shown in the tables below.

Table A1 Statistical data from Mann Whitney U test for comparison of median concentrations in period 1 and 2 in vertical trickle flow wetlands. (See section 3.2.1)

	MWU	Z (2 tailed)	Asymp. Sig	N	r
NH_3	1.00	-4.343	0.000	27	-0.836
NO_3	104.00	-1.717	0.086	36	-0.286
NO_2	101.00	-0.936	0.349	33	-0.163

Table A2. Statistical data from Mann-Whitney U test for comparison of outlet concentrations of flood/drain and submerged model wetlands. (See figure 4.1)

	MWU	Z	(Asymp. Sig (2 tailed))	N	r
Treatment 1					
TKN	364	-0.009	0.993	54	-0.012
NH_3	337	-0.467	0.640	54	-0.064
OrgN	347	-0.294	0.769	54	-0.040
NO_3	348	-0.285	0.775	54	-0.039
NO_2	356	-0.140	0.889	54	-0.019
TN	345	-0.337	0.736	54	-0.046
Treatment 2					
TKN	89	-2.112	0.035	35	-0.357
NH_3	83	-2.310	0.021	35	-0.390
OrgN	101.5	-1.700	0.089	35	-2.873
NO_3	34	-3.928	0.000	35	-0.664
NO_2	119	-1.106	0.269	35	-0.187
TN	137	-0.528	0.597	35	-0.089
Treatment 3					
TKN	31	-2.367	0.018	24	-0.483
NH_3	36	-2.078	0.038	24	-0.424
OrgN	28	-2.540	0.011	24	-0.518
NO_3	4	-3.926	0.000	24	-0.801
NO_2	39.5	-1.877	0.061	24	-0.383
TN	67	-0.289	0.773	24	-0.059

Table A3. Statistical data from Wilcoxon test for comparison of inlet and outlet concentrations in submerged and flood/drain model wetlands. See figure 4.1

	Z	Asymp. Sig (2 tailed)	N	r
Treatment 1				
TKNS	-2.547	0.011	27	-0.490
TKNF	-3.267	0.001	27	-0.629
NH_3S	-0.769	0.442	27	-0.148
NH_3F	-2.090	0.037	27	-0.402
OrgNS	-3.051	0.002	27	-0.587
OrgNF	-2.907	0.004	27	-0.559
NO_3S	-4.204	0.000	27	-0.809
NO_3F	-4.252	0.000	27	-0.818
NO_2S	-4.470	0.000	27	-0.860
NO_2F	-4.206	0.000	27	-0.809
TNS	-4.084	0.000	27	-0.786
TNF	-4.324	0.000	27	-0.832
Treatment 2				
TKNS	-3.408	0.001	15	-0.880
TKNF	-3.296	0.001	14	-0.881
NH_3S	-2.069	0.039	18	-0.488
NH_3F	-3.621	0.000	17	-0.878
OrgNS	-2.215	0.027	15	-0.572
OrgNF	-3.296	0.001	14	-0.881
NO_3S	-0.457	0.647	18	-0.108
NO_3F	-1.870	0.062	17	-0.454
NO_2S	-2.940	0.003	18	-0.693
NO_2F	-3.621	0.000	17	-0.878
TNS	-1.477	0.140	15	-0.381
TNF	-1.287	0.198	14	-0.344
Treatment 3				
TKNS	-2.981	0.003	12	-0.861
TKNF	-2.981	0.003	12	-0.861
NH_3S	-2.353	0.019	12	-0.679
NH_3F	-3.059	0.002	12	-0.883
OrgNS	-3.059	0.002	12	-0.883
OrgNF	-2.981	0.003	12	-0.861
NO_3S	-3.059	0.002	12	-0.883
NO_3F	-2.589	0.010	12	-0.747
NO_2S	-2.903	0.004	12	-0.838
NO_2F	-2.981	0.003	12	-0.861
TNS	-3.059	0.002	12	-0.883
TNF	-2.824	0.005	12	-0.815

	Z	Asymp. Sig (2 tailed)	N	r
Treatment 4				
TKN	-3.296	0.001	14	-0.881
NH_3	-3.296	0.001	14	-0.881
OrgN	-2.919	0.004	14	-0.780
NO_3	-.0.341	0.733	15	-0.088
NO_2	-2.869	0.004	15	-0.741
TN	-3.296	0.001	14	-0.881

S= submerged F = flood/drain

Tale A4 Statistical data from Mann Whitney U test for comparison of median species number in Selonda UK wetland. See Figures 5.4 and 5.5)

	MWU	Z (2 tailed)	Asymp. Sig	N	r
Comparisons by position					
in/2	126.00	-2.729	-0.006	44	-0.411
in/5	131.50	-2.602	-0.009	44	-0.395
in/out	170.00	-1.696	-0.09	44	-0.256
2/5	229.50	-.0294	0.769	44	-0.144
2/out	174.50	-1.587	0.113	44	-0.239
5/out	192.00	-1.176	0.240	44	-0.177
Comparisons by date					
01.02.06/23.03.06	14.50	-1.158	0.247	16	-0.290
01.02.06/31.05.06	24.00	0.000	1.000	16	0.000
01.02.06/07.09.06	8.00	-1.949	0.051	16	-0.487
01.02.06/14.11.06	22.00	-0.243	0.808	16	-0.061
01.02.06/25.01.07	19.00	-0.609	0.543	16	-0.152
01.02.06/20.04.07	24.00	0.000	1.000	16	0.000
01.02.06/28.06.07	14.50	-1.154	0.249	16	-0.289
23.03.06/31.05.06	47.00	-1.448	0.147	24	-0.296
23.03.06/07.09.06	41.50	-1.768	0.077	24	-0.361
23.03.06/14.11.06	49.50	-1.308	0.191	24	-0.267
23.03.06/25.01.07	43.00	-1.679	0.093	24	-0.343
23.03.06/20.04.07	57.00	-0.869	0.386	24	-0.177
23.03.06/28.06.07	23.50	-2.810	0.005	24	-0.574
31.05.06/07.09.06	26.50	-2.640	0.008	24	-0.539
31.05.06/14.11.06	65.50	-0.376	0.707	24	-0.077
31.05.06/25.01.07	64.00	-0.464	0.643	24	-0.095
31.05.06/20.04.07	68.50	-0.203	0.839	24	-0.041
31.05.06/28.06.07	51.50	-1.186	0.236	24	-0.242
07.09.06/14.11.06	29.50	-2.460	0.014	24	-0.502
07.09.06/25.01.07	26.00	-2.662	0.008	24	-0.543
07.09.06/20.04.07	34.50	-2.171	0.030	24	-0.443
07.09.06/28.06.07	12.50	-3.441	0.001	24	-0.702
14.11.06/25.01.07	59.00	-0.752	0.452	24	-0.154
14.11.06/20.04.07	71.50	-0.029	0.977	24	-0.006

Comparisons by date (continued)

	MWU	Z (2 tailed)	Asymp. Sig	N	r
14.11.06/28.06.07	42.00	-1.737	0.082	24	-0.355
25.01.07/20.04.07	64.00	-0.463	0.643	24	-0.095
25.01.07/28.06.07	57.00	-0.868	0.386	24	-0.177
20.04.07/28.06.07	47.00	-1.446	0.148	24	-0.295

Table A5. Statistical data from Mann Whitney U test for comparison of inlet and outlet concentrations in Selonda UK wetland B and C. See figure 6.1

Wetland B Phase 1 submerged					
NH_3U	6.00	-1.928	0.054	12	-0.557
NH_3L	14.50	-0.572	0.568	12	-0.165
NH_3E	2.00	-2.571	0.010	12	-0.742
NO_2U	4.00	-2.246	0.025	12	-0.648
NO_2L	13.50	-0.724	0.469	12	-0.209
NO_2E	2.00	-2.567	0.010	12	-0.741
NO_3U	1.50	-2.647	0.008	12	-0.764
NO_3L	13.00	-0.815	0.415	12	-0.235
NO_3E	2.00	-2.585	0.010	12	-0.746
Wetland C Phase 1 submerged					
NH_3U	6.00	-1.939	0.053	12	-0.560
NH_3L	16.00	-0.332	0.740	12	-0.096
NH_3E	7.00	-1.768	0.077	12	-0.510
NO_2U	0.00	-2.903	0.004	12	-0.838
NO_2L	5.50	-2.019	0.043	12	-0.583
NO_2E	2.50	-2.486	0.013	12	-0.718
NO_3U	1.50	-2.690	0.007	12	-0.854
NO_3L	7.50	-1.440	0.150	11	-0.434
NO_3E	3.00	-2.211	0.027	11	-0.667
Wetland B Phase 2 submerged					
NH_3U	2.00	-2.200	0.028	10	-0.696
NH_3L	9.00	-0.740	0459	10	-0.234
NH_3E	0.00	-2.611	0.009	10	-0.826
NO_2U	4.00	-1.776	0.076	10	-0.562
NO_2L	11.50	-0.211	0.833	10	-0.067
NO_2E	4.00	-1.776	0.076	10	-0.562
NO_3U	2.00	-2.227	0.026	10	-0.704
NO_3L	10.50	-0.430	0.667	10	-0.136
NO_3E	2.50	-2.108	0.035	10	-0..667

Wetland C Phase 2 submerged					
NH_3U	0.00	-2.611	0.009	10	-0.826
NH_3L	12.00	-0.104	0.917	10	-0.033
NH_3E	0.00	-2.611	0.009	10	-0.826
NO_2U	5.00	-1.567	0.117	10	-0.496
NO_2L	9.00	-0.733	0.463	10	-0.232
NO_2E	4.00	-1.781	0.075	10	-0.563
NO_3U	6.50	-1.265	0.206	10	-0.400
NO_3L	10.00	-0.530	0.596	10	-0.168
NO_3E	9.00	-0.736	0.462	10	-0.233
Wetland B Phase 3 flood/drain					
NH_3U	4.00	-1.155	0.248	8	-0.408
NH_3L	6.00	-0.577	0.564	8	-0.204
NH_3E	0.00	-2.309	0.021	8	-0.816
NO_2U	4.00	-1.155	0.248	8	-0.408
NO_2L	0.00	-2.309	0.021	8	-0.816
NO_2E	0.00	-2.309	0.021	8	-0.816
NO_3U	8.00	0.00	1.000	8	0.000
NO_3L	6.00	-0.615	0.538	8	-0.217
NO_3E	7.50	-0.146	0.884	8	-0.052
Wetland C Phase 3 flood/drain					
NH_3U	2.00	-1.732	0.083	8	-0.612
NH_3L	6.00	-0.592	0.554	8	-0.209
NH_3E	0.00	-2.323	0.020	8	-0.821
NO_2U	2.50	-1.597	0.110	8	-0.565
NO_2L	0.00	-2.232	0.020	8	-0.821
NO_2E	2.50	-1.607	0.108	8	-0.568
NO_3U	2.50	-1.607	0.108	8	-0.568
NO_3L	5.00	-0.871	0.384	8	-0.308
NO_3E	5.00	-0.871	0.384	8	-0.308

U= upper cell, L= lower cell, E= entire wetland

Table A6. Statistical data from Mann Whitney U test for comparison of median outlet concentrations of wetland B with wetland C at the Selonda UK site. See figure 6.1

	MWU	Z (2 tailed)	Asymp. Sig	N	r
Phase 1 Upper wetland outlet					
NH_3	18.00	0.00	1.000	12	0.000
NO_2	12.05	-0.915	0.360	12	-0.264
NO_3	18.00	0.00	1.000	12	0.000
Phase 2 Upper wetland outlet					
NH_3	12.00	-0.106	0.916	10	-0.034
NO_2	7.50	-1.048	0.295	10	-0.331
NO_3	10.5 0	-0.422	0.673	10	-0.133

Phase 3 Upper wetland outlet					
NH_3	5.50	-0.726	0.468	8	-0.257
NO_2	0.00	-2.309	0.021	8	-0.816
NO_3	0.00	-2.337	0.019	8	-0.826
Phase 1 Lower wetland outlet					
NH_3	14.00	-0.652	0.514	12	-0.188
NO_2	16.00	-0.322	0.747	12	-0.093
NO_3	10.50	-0.845	0.398	12	-0.244
Phase 2 Lower wetland outlet					
NH_3	10.00	-0.522	0.602	10	-0.165
NO_2	12.00	-0.106	0.916	10	-0.034
NO_3	6.00	-1.379	0.168	10	-0.
Phase 3 Lower wetland outlet					
NH_3	4.00	-1.162	0.245	8	-0.367
NO_2	0.50	-2.191	0.028	8	-0.693
NO_3	3.00	-1.488	0.137	8	-0.471

Tale A7 Statistical data from Mann Whitney U test for comparison of inlet and outlet concentrations in Selonda UK wetland A. See figure 6.3

	MWU	Z	Asymp. Sig (2 tailed)	N	r
sumerged					
NH_3U	5.00	-0.877	0.381	8	-0.310
NH_3L	1.00	-2.021	0.043	8	-0.716
NH_3E	1.00	-2.021	0.043	8	-0.716
NO_2U	2.00	-1.742	0.081	8	-0.616
NO_2L	7.50	-0.149	0.882	8	-0.053
NO_2E	1.50	-1.888	0.059	8	-0.668
NO_3U	3.50	-1.340	0.180	8	-0.474
NO_3L	5.50	-0.764	0.445	8	-0.270
NO_3E	2.00	-1.764	0.078	8	-0.624
flood/drain					
NH_3U	4.00	-3.477	0.001	20	-0.777
NH_3L	39.00	-0.832	0.406	20	-0.186
NH_3E	6.00	-3.326	0.001	20	-0.744
NO_2U	9.00	-3.107	0.002	20	-0.695
NO_2L	22.00	-2.138	0.032	20	-0.478
NO_2E	5.50	-3.382	0.001	20	-0.756
NO_3U	18.50	-2.388	0.017	20	-0.534
NO_3L	19.00	-2.354	0.019	20	-0.526
NO_3E	2.50	-3.612	0.000	20	-0.808

U= upper cell, L= lower cell, E= entire wetland

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